

A NOVEL MECHANISM FOR HUMAN PAPILLOMAVIRUS MEDIATED
TUMORIGENESIS: EXAMINING A ROLE FOR HPV E6 PROTEIN IN CYLD
MEDIATED NF- κ B ACTIVATION

by

Charlie Vincent Shaw Junior

University Program in Genetics and Genomics
Duke University

Date: _____

Approved:

Kenneth A. Alexander, Supervisor

Kenneth Kreuzer

Gary Cox

David Pickup

William Phelps

Dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in the
University Program in Genetics and Genomics
in the Graduate School of Duke University.

2009

ABSTRACT

A NOVEL MECHANISM FOR HUMAN PAPILLOMAVIRUS MEDIATED
TUMORIGENESIS: EXAMINING A ROLE FOR HPV E6 PROTEIN IN CYLD
MEDIATED NF- κ B ACTIVATION

by

Charlie Vincent Shaw Junior

University Program in Genetics and Genomics
Duke University

Date: _____

Approved:

Kenneth A. Alexander, Supervisor

Kenneth Kreuzer

Gary Cox

David Pickup

William Phelps

An abstract of a dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
University Program in Genetics and Genomics
in the Graduate School of Duke University.

2009

Copyright by
Charlie Vincent Shaw Junior
2009

Abstract

Human papillomavirus (HPV) infection of mucosal epithelium by ‘high-risk’ HPV types has a prominent role in the development of anogenital intraepithelial neoplasias and carcinomas. Human epithelial cells transformed with the HPV E6 oncoprotein survive even under conditions that normally lead to cell apoptosis. This phenomenon has been attributed to HPV E6’s ability to promote the degradation of the tumor suppressor protein p53. More recently, it has been demonstrated that HPV E6 contributes to activation of the NF- κ B pathway. NF- κ B is a transcription factor involved in the regulation of genes associated with cellular proliferation, apoptosis and inflammatory responses. In addition to p53 suppression, HPV E6 modulation of NF- κ B activation presents another mechanism for HPV-driven tumorigenesis. However, it was not known how HPV E6 promotes NF- κ B pathway activation. To address how HPV E6 leads to NF- κ B activation, we identified an association between HPV E6 and the human cylindromatosis gene product (CYLD). CYLD is an endogenous inhibitor of canonical NF- κ B activation. We showed that HPV E6 proteins could precipitate CYLD *in vitro* using a co-immunoprecipitation assay. Demonstrating that HPV E6 and CYLD proteins bind each other raised the possibility that this binding relationship would have a functional effect upon the NF- κ B pathway by altering CYLD-mediated suppression of NF- κ B activation. To identify HPV E6 functional relationship with CYLD and to determine how HPV E6 activates the NF- κ B pathway, we transfected cells with either HPV E6 expression

vectors containing the high-risk HPV type 16 E6 or the low-risk HPV type 11 E6 along with a CYLD expression vector. We showed HPV16 E6 expression in 293 cells blocked the ability of CYLD to inhibit CD40 ligand-stimulated NF- κ B activation. Interestingly, HPV11 E6 was unable to inhibit CYLD mediated suppression of NF- κ B in our system. CYLD had previously been shown to suppress NF- κ B activation by removing stimulatory lysine 63-linked ubiquitin chains from TRAF2. We found CYLD expression in 293 cells leads to dose-dependent reduction in TRAF2 levels. This CYLD-mediated loss of TRAF2 is inhibited by co-expression of high-, but not low-risk E6 proteins. It was known that CYLD phosphorylation *in vivo* suppresses CYLD deubiquitination actions of canonical pathway proteins; we therefore tested the extent of CYLD phosphorylation when co-expressed with HPV E6, and discovered that CYLD phosphorylation was increased in the presence of HPV E6. This compilation of experiments suggests that with HPV16 E6 binding to CYLD, the E6 protein blocks CYLD-mediated TRAF2 loss and thereby TRAF2 is available to activate the canonical NF- κ B pathway. Blocking HPV E6-mediated NF- κ B activation may prove beneficial as a means for designing therapies that inhibit HPV-mediated tumorigenesis. The differences we detected between HPV11 E6 and HPV16 E6 are supported by other studies that showed E6 protein variations account for molecular and clinical differences among HPV infection outcomes. Similarly, there exist intratype E6 variations in HPV16. We obtained cervical specimens from patients with cytopathogenic changes consistent with the onset of cervical dysplasia infected with HPV16 E6 and the human immunodeficiency virus. We hypothesized

the immunocompromised individual may harbor unique HPV16 E6 variants. Using PCR detection methods to amplify the HPV16 E6 DNA and sequencing technology, we identified that some of the samples indeed had nucleotide polymorphisms, resulting in amino acid sequence changes. However, the HPV E6 variants we detected were previously described, and can be classified into known geographic HPV clades. Some of the HPV E6 variants we observed are suggested to be associated with progression to cervical cancer, but further evaluation is required.

This work is dedicated to the memory of my grandparents:

Mallie & Cora Lee Shaw, wisdom gleamed from Br'er Rabbit will always

be my guide;

and Walter & Lelia Roberts, whom I never met, but know their prayers and sacrifices

supersede time.

And to my parents:

Charlie Vincent Shaw & Ruth Esther Shaw

Charlie Vincent Shaw transitioned from this earth to glory during the writing of this

dissertation. He was always an advocate for me and my number one fan. If I ever

needed anything, he provided it, even without a 'money tree' in the back yard. I miss

you, daddy. Ruth Esther Shaw shed tears every single time we parted ways. I think

about her and recognize, home is where my greatest source of earthly strength

resides. I love you both.

And to my sisters:

Miranda Velma Shaw Martin aka "Miss Parker" started the lamp of learning. Lora

Ann Shaw McGougan with her twenty-dollar bill crumbled and placed in the palm of

my hand kept it going. And Lynne Deanna Shaw Tull provided a consent reminder

that if I needed anything, she was only a phone call away.

Table of Contents

Abstract.....	iv
List of Tables.....	ix
List of Figures.....	x
List of Abbreviations.....	xi
Acknowledgements.....	xiv
I. Introduction of Human Papillomavirus.....	1
II. NF- κ B Background.....	25
III. The HPV E6 protein binds CYLD and modulates NF- κ B.....	42
IV. HPV E6 rescues CYLD-mediated TRAF2 loss.....	63
V. Genetic variability in the HPV E6 open reading frame of HPV16 and HIV infected adolescents.....	88
VI. Discussion and future directions.....	106
References.....	121
Biography.....	139

List of Tables

Table I-1. The HPV genes and the function of their gene products....	9
Table II-1. NF- κ B family members.....	30
Table V-1. Nucleotide comparison of the different HPV16 E6 gene polymorphisms.....	98
Table V-2. Identified HPV16 E6 amino acid variations.....	100

List of Figures

Figure I-1	Electron micrograph image of HPV showing virion morphology.....	5
Figure I-2	Schematic representation of the HPV genome.....	7
Figure I-3	Cervical intraepithelial neoplasia staging.....	16
Figure I-4	Classical biochemical actions of HPV oncogenic proteins E7 and E6.....	23
Figure II-1	Classical NF- κ B pathway.....	34
Figure II-2	Schematic representation of CYLD protein.....	40
Figure III-1	Characterization of HPV16 E6 and HPV11 E6 effects on NF- κ B activation.....	50
Figure III-2	HPV16 E6 blocks the ability of CYLD to suppress NF- κ B activation in cells deficient in p53.....	54
Figure III-3	The HPV E6 protein binds CYLD.....	57
Figure IV-1	CYLD alters TRAF2 protein levels.....	69
Figure IV-2	Silencing of endogenous E6 expression in SiHa cells leads to loss of TRAF2.....	73
Figure IV-3	HPV E6 proteins increase CYLD phosphorylation.....	76
Figure IV-4	CYLD gene transcription levels vary between HPV-infected and non-HPV infected cells.....	79
Figure IV-5.	HPV E6 protein expression increase CYLD	82
Figure V-1	Primer sequences used for PCR amplification of the HPV 16 E6 gene.....	94
Figure VI-1	Schematic showing HPV16 E6 involvement in NF- κ B signaling pathway.....	114

List of Abbreviations

- 293T- Human embryonic kidney cell line
- Bcl-3- B-cell lymphoma 3-encoded protein
- C33A- non-human papillomavirus transformed cervical cell line
- CD40- TNF receptor superfamily member 5
- CD40L- CD40 ligand
- CIN- cervical intraepithelial neoplasia
- CYLD- cylindromatosis protein
- DNA- deoxyribonucleic acid
- DNase- deoxyribonuclease
- E1- the papillomavirus early gene 1
- E2- the papillomavirus early gene 2
- E4- the papillomavirus early gene 4
- E5- the papillomavirus early gene 5
- E6- the papillomavirus early gene 6
- E6-AP- E6 associated protein
- E7- the papillomavirus early gene 7
- EV- epidermodysplasia verruciformis
- GAPDH – glutaraldehyde phosphate dehydrogenase
- HeLa- a cell line derived from a cervical carcinoma
- HIV- human immunodeficiency virus

HPV- human papillomavirus

HPV 16R- prototype HPV 16 sequence

IKK- I κ B kinase

κ B- nine or ten base pair DNA sequence recognized by NF- κ B

L1- the papillomavirus late gene 1

L2- the papillomavirus late gene 2

mCi- milli-curie

mL- milliliter

mRNA- messenger RNA

nM- nanomolar

NEMO- IKK gamma (NF- κ B essential modulator protein)

NF- κ B- nuclear factor κ B

NIK- NF- κ B-inducing kinase

PBS- phosphate buffered saline

PCR- polymerase chain reaction

Rb- retinoblastoma gene

REL- reticulo endotheliosis

RHD- REL homology domain

RNA- ribonucleic acid

RNAi- RNA interference

RT-PCR- reverse transcriptase polymerase chain reaction

SDS-PAGE- sodium dodecyl sulfate- polyacrylamide gel electrophoresis

SiHa- a cell line derived from a cervical carcinoma

siRNA- short interfering RNA

Sp1- Sp1 transcription factor

SV40- simian virus 40

TBP- TATA box binding protein

TLR- toll like receptor

TNF- tumor necrosis factor

TNF- α - tumor necrosis factor alpha

TNFR- tumor necrosis factor receptor

TRIP- tumor necrosis factor receptor associated factor-interacting protein

W12-E- a cell line derived from a cervical dysplasia

μg - microgram

μL - microliter

μM - micromolar

Acknowledgements

This dissertation represents my efforts in the laboratory of Kenneth Andrew Alexander, MD, Ph.D. His continued support and patient disposition allowed me to learn and grow in the best possible environment. Throughout the challenges faced, I never feared as I knew he would have a positive perceptiveness to share and a comforting ear to lend. I would like to thank all the lab members: Xiaohua Zhang, Ph.D., who always seemed to know the answer and have the reagent, Shuhan Yu, as her friendly smile made lab life enjoyable; and to the numerous scholars who spent time in the lab, Brandon Hatcher, Katherine Kenerson, Frank Aguilar, and Jesus Correa, each of whom has been directly involved in the work presented in this dissertation. Additionally, I would like to recognize Kathleen McKenna, MD., for her contributions to the data in the genetic variability of E6 open reading frame chapter.

I. Introduction of Human Papillomavirus

Historical overview of human papillomavirus

The wart, a benign growth of epithelial tissue, has been noted in medical related literature for centuries, with physicians such as Hippocrates defining condyloma acuminatum as pointed round swelling around the anogenital region and Celsus identifying three types of skin warts in his *De Medicina* of 25AD. In the late nineteenth century and early twentieth century, scientist J.F Payne identified the infectious nature of common warts¹ and G. Ciuffo observed papilloma lesions resulting from the inoculation of wart tissue into skin suggesting the transmissibility of papilloma agents (reviewed in ²). But it was not until the 1960's and 1970's that the explosion of research broadened the understanding and impact of human papillomavirus (HPV) related diseases. Around 1975, Harold zur Hausen made the claim for HPV being a plausible cause of cervical cancer³. Viral particles were detected in vulva warts⁴ and cervical condylomatous lesions⁵ and laryngeal papillomas⁶, contributing to similar observations made by others such as Dunn and Ogilvie⁷, suggesting a viral pathogen in human warts. Koilocytotic cells were described in flat epithelial lesions and hypothesized to be the initial manifestations of dysplasia caused by HPV⁸. This hypothesis was validated when the koilocytes in pre-neoplastic cervical lesions⁵ and koilocytes in invasive cervical cancer⁹ were shown to contain HPV viral particles. Harold zur Hausen was awarded the Nobel Prize in Medicine in 2008 for his contribution to the current understanding of HPV disease.

HPV Virology

Papillomaviruses, or *Papillomaviridae*, are a family of viruses that infect many members of the animal kingdom, including humans, monkeys, horses, cattle, deer, rabbits, dogs, and mice¹⁰. Despite a broad phylogenetic distribution, papillomaviruses are host specific and will not infect across species. The virus that infects humans, HPV, is a non-enveloped, icosahedral, circular double-stranded DNA virus that invades epithelial cells (Fig. I-1 and Fig. I-2)¹¹. This virus is relatively small, containing a genome of approximately eight kilobases. HPV has eight identified open reading frames encoding six early genes: E1, E2, E4, E5, E6, E7 and two late genes: L1 and L2 (Table I-1). The early genes encode proteins important for viral replication and cellular transformation. Early product proteins are detected in proliferating areas of HPV-induced lesions. Late genes code for structural proteins, and are detected in differentiating parts of the epithelium where productive viral DNA replication occurs¹².

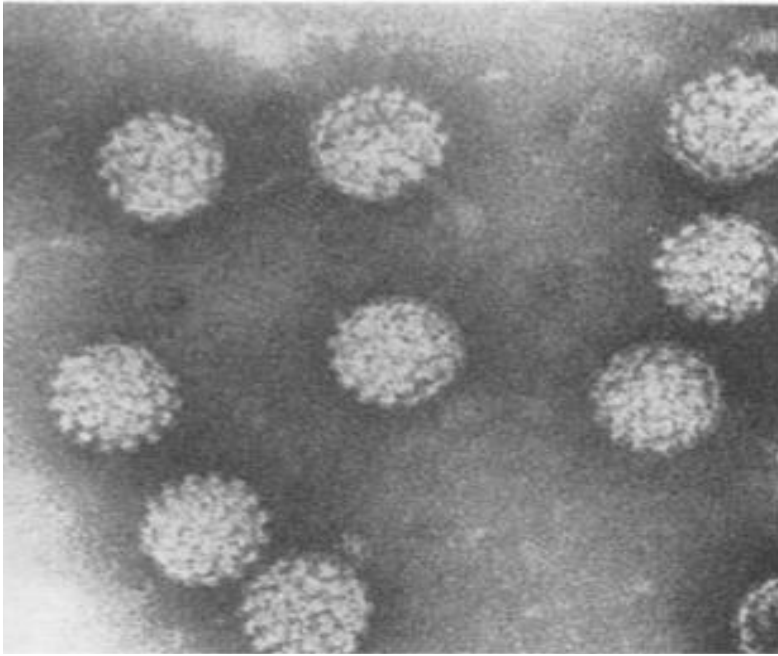
The E1 and E2 viral proteins are involved in viral genome replication¹³. E1 is a 3' to 5' DNA helicase with sequence similarities with the well studied simian virus 40 (SV40) large TAg and the parvovirus helicase NS1. E1 is also involved in recruiting host cellular replication proteins to the viral origin of replication. The E2 protein is a transcriptional *trans*-modulator that contains an amino terminal trans-activation domain, a poorly conserved hinge domain and a DNA binding carboxyl-

terminal domain. E2 is involved in recruiting E1 protein to the viral replication origin to promote origin-dependent viral DNA replication. E2 also interacts with several host cell transcription factors, including Sp1 and TBP. The E4 protein is believed to promote viral release from host cell by disrupting the cytokeratin network¹⁴. The E5 protein is a membrane bound protein that enhances cellular growth by interacting with the epidermal growth factor receptors¹⁵. E6 and E7 are the viral oncoproteins responsible for virus-induced cell transformation.

Over 120 different types of HPV have been cloned and sequenced. HPV typing is based on homology of L1, E6 and LCR sequences, rather than on serology, because HPV capsid proteins are highly conserved. Generally, different HPV types have less than ninety percent homology. HPV types are rigidly epitheliotropic, meaning a particular HPV type will only infect keratinocytes of either cutaneous or mucosal epithelium. Cutaneous HPV infections may be manifested as proliferating lesions, commonly known as warts¹⁶. HPV types that infect mucosal epithelial cells, such as cells found in the lower regions of the anogenital tract, and the upper respiratory tract, are subdivided into 'low-risk' and 'high-risk' categories based upon oncogenic potential. DNA from low-risk HPV types (such as types 6 and 11) are commonly found in benign lesions of the anogenital tract (genital warts) and laryngeal papillomas. High-risk types (including types 16 and 18) are detected in anogenital malignancies^{17,18}, including cervical intraepithelial neoplasia¹⁹, squamous cell carcinoma¹⁹, cervical carcinoma¹⁶ and adenocarcinoma¹⁶.

Figure I-1. Electron micrograph image of HPV showing virion morphology.

Electron microscopy was used by J.D. Oriel and June D. Almeida to show viral particles in vulval warts.



← HPV virion

Figure I-2. Schematic representation of the HPV genome. Depicted is the HPV16 genome, a circular double strand DNA molecule. The open reading frames are shown as darkened fragments. LCR is the long control. The LCR contains the major early promoter, enhancer sequences, and the origin of replication.

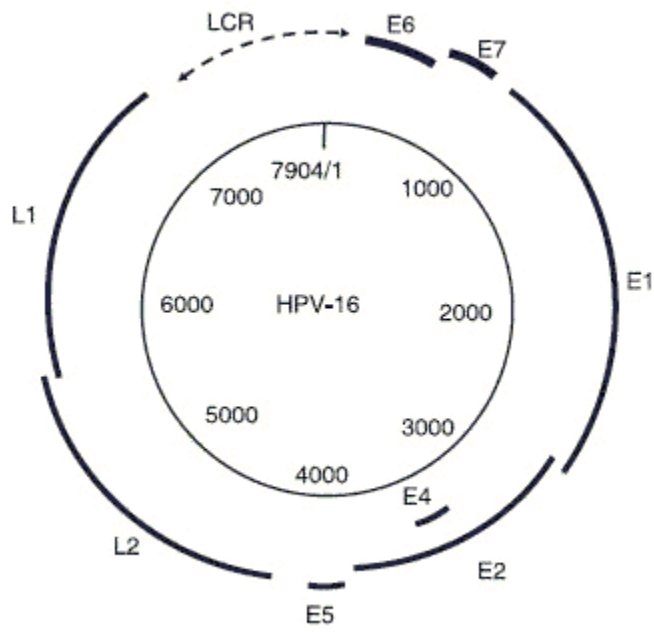


Table I-1. The HPV genes and the function of their gene products. HPV has six early (E) and two late (L) genes. The E regions encode non-structural proteins while the L regions encode structural proteins.

	Protein Function
HPV gene	
E1	3' to 5' DNA helicase. Sequence similarities with SV40 large TAg and the parvovirus helicase. Required for HPV replication. Binds the p68 subunit of DNA polymerase α .
E2	Transcriptional <i>trans</i> -modulator. Binds DNA at a specific palindromic sequence present in several places within and outside of the HPV <i>ORI</i> . Recruits E1 protein for origin-dependent viral DNA replication. Interacts w/several host cell transcription factors (including Sp1, TBP). Contains an amino terminal trans-activation domain, hinge and carboxyl-terminal DNA binding domain.
E4	Late expression protein. May induce collapse of cyokeratin network, potentially to enhance virion release. Only moderately conserved among HPV types.
E5	Viral oncoprotein. An 84 amino acid, hydrophobic, membrane associated protein. DNA sequence not well conserved among HPV types and animal PVs. Appears to up-regulate the activity of cellular growth factor receptors.
E6	Viral oncoprotein. Inhibits host cell apoptosis by binding host tumor suppressor cell p53. The E6:p53 complex targets the p53 protein for ubiquitin pathway-mediated degradation.
E7	Oncoprotein that binds to host cell pRb family tumor suppressors, leading to the release of sequestered E2F transcription factor resulting in the movement of the host cell through G1/S transition and reentry into cell cycle.
Late protein 1	Major capsid protein.
Late protein 2	Minor capsid protein.

HPV Lifecycle

HPV enters the body through micro-lesions caused by minor trauma such as occurs with sexual intercourse, or through skin abrasion breaking through the epidermis. Upon entrance, HPV infects basal epithelial cells. The host cell surface receptor for HPV is unknown; however, the integrin alpha-six beta-four protein complex, expressed during lesion repair, is a potential candidate¹². HPV can undergo nonproductive and productive replication phases. Once an infection is established in the host basal keratinocytes, it is predicted that HPV nonproductive replication maintains a copy number of 50-100 genomes per host progeny cell²³. It is also predicted that nonproductive replication continues the source of HPV episomes within non-differentiating basal epithelial cells that are undergoing controlled cell division. HPV viral proteins appear not to be essential for nonproductive replication, according to work completed by Angeletti *et al*²⁰. These investigators discovered that the human papillomavirus full-length type 16 (HPV16) genome, when linked in *cis* to a selectable yeast marker gene, could replicate stably as an episome in *Saccharomyces cerevisiae* without the supplement of any HPV proteins, including the viral E1 protein (termed E1-independent replication)²¹.

During normal keratinocyte division, one daughter cell is retained in the basal layer and the other will exit the basal layer to move up through the epidermal layers and undergo terminal squamous differentiation to become a mature skin cell. In HPV infected cells, differentiation coincides with explosive HPV productive viral replication. The daughter cell designated to become a mature skin cell undergoes

differentiation and abandons cell division, posing a potential problem for HPV in the differentiating cell. Terminally differentiated squamous cells not undergoing cell division stop synthesis of DNA replication proteins, thereby setting up conditions which do not support HPV viral replication. The virus overcomes this dilemma by using viral proteins to stimulate a G1 to S phase cell-cycle transition in host cells, allowing initiation of productive viral amplification. In the replication active differentiating keratinocyte, productive HPV DNA replication results in the exponential synthesis of HPV DNA and capsid proteins. Thus, keratinocyte differentiation is absolutely required for successful completion of HPV's lifecycle. The newly synthesized viral DNA is assembled into progeny virions and the infectious virion-containing cells are sloughed off from the skin surface. Virions can be transmitted directly to other individuals. While close physical person to person contact is the dominant mode of transmission for adults, transmission from mother to child during vaginal birth has been reported in laryngeal papillomatosis cases²³.

Clinical relevance of HPV

Although warts have been recognized as a human problem since ancient times; and while HPV infections in skin, such as the common wart (*verruca vulgaris*), flat warts and plantar warts are the most widely recognizable manifestations, warts are not generally associated with major health risks (with exceptions among the immunocompromised were in rare cases epidermodysplasia verruciformis has been reported). HPV is a globally prevalent infectious disease,

with upwards of 75% of the reproductive-age population infected with sexually transmitted HPV²². Mucosotropic (infecting mucus lined epithelium of the penis, vagina, anus, and oral and respiratory tract) HPV types, of the low-risk types most commonly result in anogenital warts (*condyloma accuminata*). Although these lesions rarely progress into malignancies, the symptoms of low-risk HPV disease including pain, discomfort and stress related to avoidance of closeness, can cause distress in patients. Recurrent respiratory papillomatosis (persistent laryngeal papillomas) can occur in children infected during vaginal birth or by adults engaging in oral sex. Papilloma masses can grow to block airway passages, leading to death by suffocation. Thus, repeated surgeries are often needed to remove the papillomas.

Infection with high-risk HPV types is more daunting because in a small percentage of cases, infection can persist and progress towards high-grade dysplasias and carcinomas, most notably cervical, penile and anal cancers. This malignant progression of HPV infection can occur because high-risk HPVs are generally successful at evading clearance by the immune system. HPV infections can remain latent for years. Reactivation can occur.

The mucosal epithelium of both sexes is susceptible to HPV infection. The female cervix contains a transitional area located between the squamous epithelium of the exocervix and the columnar epithelium of the endocervical canal, known as the transformation zone. The transformation zone contains immature, metaplastic and hormonally responsive cells, making this region highly susceptible to HPV and to production of malignant cells²³. Persistent cervical infection with certain types of

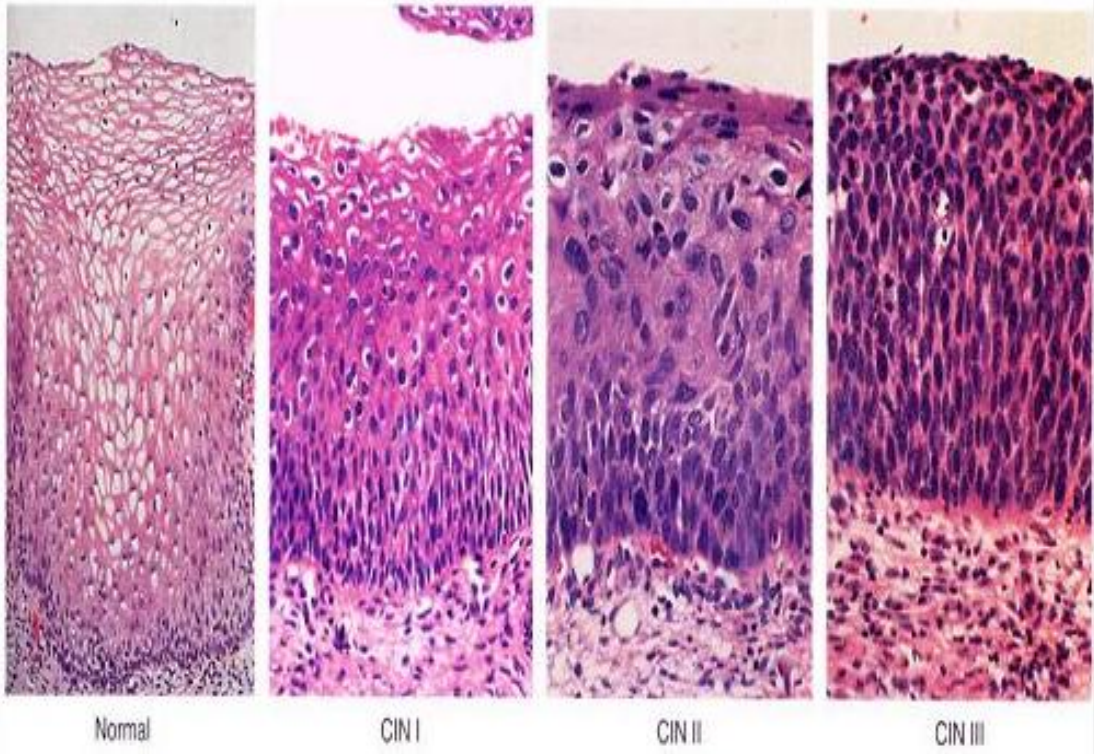
HPV is the single most important risk factor for cervical carcinoma. By identifying HPV DNA in samples from women from over twenty different countries, it is extrapolated that the prevalence of HPV DNA in cervical cancers worldwide is approximately 99.7%^{24,25}. HPV types 16, 18, 31, 33 and 45 have been detected in 63-97% of invasive cervical carcinomas, with HPV 16 and 18 being the most widely detected^{26,27}.

Cervical intraepithelial neoplasia (CIN) characterizes the histological changes that occur to the cervix as a result of HPV disease progress. CIN is diagnosed using biopsies of tissue obtained during a colposcopy. CIN is recognized by disturbances of cellular maturation, stratification and cytological atypia (Fig. I-3). In CIN, grades range from 1-3, with grade 3 being characterized as a population of cells with large, hyperchromatic, rather pleomorphic nuclei and very little cytoplasm occupying the full thickness of the epithelium. It is estimated that over 50% of CIN 1 lesions will regress spontaneously, as well as 40% of CIN II lesions²⁸. Treatment for CIN includes removal of abnormal cells. Although it is unlikely that even CIN III lesions will advance to carcinoma, in total nearly 400,000 new cases of invasive cervical cancer are diagnosed worldwide each year, and approximately 11,000 new cases in the United States. Cervical cancer claims the lives of nearly 4000 mothers, wives, daughters and sisters in American each year^{29,30}.

Central and South America, Africa and the Caribbean are areas of increased cervical cancer risk³⁰. Within the United States, there are higher incidences of cervical cancer in African-Americans and Hispanics women when compared with

their Caucasian counterparts. Black women had 57% five year survival rates compared to 71% five year survival rates for white women³⁰. The current hypothesis relates this disparity to socioeconomic dissimilarities.

Figure I-3. Cervical intraepithelial neoplasia staging. Histological changes occur to the squamous cells of the cervix during the progression of dysplasia. Pictured is epithelial cells from normal to CIN I through CIN III. Image provided courtesy of Talaat S. Tadros MD, Emory University School of Medicine (adapted from Robbins Pathologic Basis of Disease, 6th Edition, pg10051).



HPV treatment and prevention

The cost burden estimates associated with genital HPV infections in the US suggest an annual expense as high as \$6 billion dollars, making genital HPV the second most costly STD after HIV infection³¹. Treatment regimens for cervical cancer include surgery, radiation therapy, and chemotherapy. A concern faced by health officials is that the majority of HPV infections are frequently missed in physical exams because HPV infection is usually asymptomatic and is only detectable by the use of HPV DNA detection tests³². Use of regular Papanicolaou tests (Pap smears) has drastically improved detection of HPV associated diseases. Increasingly, identification of an abnormal Pap smears results in the ordering of a HPV DNA test by clinicians.

Developing antiviral drugs for treatment of HPV has posed a challenge. Drug candidates that claim to inhibit some DNA/RNA viruses have emerged, but none of these candidates have been highly successful against HPV³². Recently, two HPV vaccines have been developed that show high promise of reducing the prevalence of genital warts, cervical dysplasia and cervical cancer in previously uninfected individuals. *Gardasil*, a quadrivalent vaccine (containing antigen from HPV 16, 18, 6, and 11) manufactured by Merck has received FDA approval and is available in the US. A second bivalent vaccine containing HPV 16 and 18 antigens has been developed by GlaxoSmithKline. Both are recommended for females before the onset of sexual activity.

HPV Oncogenesis

HPV DNA is normally maintained as a separate episome within the host cell. However, during a process currently not well characterized, viral DNA can integrate into the host cell DNA. Although the site of integration within the human genome appears to be random, E1 or the E2 coding sequences are generally disrupted^{33,34,35}. In the intact genome, the E2 gene product normally suppresses E6 and E7 expression; however, disruption of E2 expression results in constitutive expression of E6 and E7. The E6 and E7 of high-risk HPV types working in concert are adequate for the transformation and immortalization of keratinocytes^{36,37}. Along with E5, E6 and E7 proteins function to effectively offset cellular immune system surveillance and block apoptotic signals.

The HPV E7 proteins of high-risk HPV types contribute to cell transformation through HPV E7 involvement with cellular growth and proliferation control. An early identified target of HPV E7 is the retinoblastoma tumor suppressor protein (Rb) and related proteins p107 and p130³⁸. The pRb protein functions as a cell cycle regulator by controlling the G1 to S transition. In HPV uninfected cells not actively proliferating, hypophosphorylated Rb is complexed with histone deacetylase to bind the E2F family of transcription factors (E2F1 through E2F5) preventing their stimulatory growth effects by halting G1/S transition of the cell cycle. HPV E7 preferentially binds to the hypophosphorylated Rb/histone deacetylase complex, and by doing so, displaces pRb bound E2F. Ultimately, the E7/Rb interaction leads to proteasome-mediated degradation of pRb³⁹ (Fig. I-4a). Cyclin A and cyclin E, which

activate cdk2, are two genes transcriptionally up-regulated by the unbound E2F. Cyclin E/cdk2 is essential to S phase entry and cyclin A/cdk2 promotes the S phase to M phase transition. Thus, the release of E2F allows for cdk independent cell cycle progression, resulting in cellular proliferation^{38,40}. HPV E7 proteins also directly bind additional proteins involved in cell cycle, such as the cyclin A and E^{41,42}, cyclin-dependent kinase inhibitor p21^{CIP1} protein^{43,44}, and protein phosphatase 2A (PP2A)^{45 46}. PP2A interacts with protein kinase P (PKB) or Akt. Sequestering of PP2A by HPV E7 interrupts this interaction allowing for anti-apoptotic signaling by PKB/Akt⁴⁵.

The customary cellular response mechanism to abnormal proliferation (outside of normal physiological and signal-dependent means), and other exogenous and endogenous cellular stressors is the up-regulation of pro-apoptotic factors, including the tumor suppressor protein p53. HPV E6 has been shown to inhibit apoptosis by p53 and other pro-apoptotic factors such as Bak and Myc. HPV E6 forms a complex with the cellular ubiquitination enzyme E6 associated protein (E6AP), and this E6/E6AP complex functions as an ubiquitin ligase to facilitate p53, Bak and Myc proteasome degradation (Fig. I-4b). A normal p53 half-life of several hours in keratinocytes is reduced to less than 20 minutes in the presence of HPV E6^{47,48}.

The difference between high-risk and low-risk HPV types ability to effectively transform keratinocytes may be attributed to properties among the

oncogenic proteins in part. Low-risk HPV E7 proteins have been shown to bind Rb with lower efficiency and HPV-1 E7 was unable to induce Rb degradation^{49,50}.

Similar to the difference observed between high-risk and low-risk E7 types, low-risk HPV E6 proteins are unable to drive proteasomal degradation of p53⁵¹. Tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)-mediated apoptosis⁵², TNF-mediated apoptosis⁵³ and Fas-mediated apoptosis⁵⁴ have all been shown to be blocked by high-risk HPV E6 interaction with accessory proteins within these signaling pathways.

High-risk HPV E6, but not low-risk HPV E6, has also been demonstrated to effectively bind and hinder co-activators of various transcription factors and transcription accessory proteins, such as p300 and CPB, each of which is involved in cell cycle progression and growth⁵⁵. HPV16 E6 can alter transcription factors directly as seen in HPV E6 inducing consensus site binding by the transcription factor NF- κ B⁵⁶.

Additionally, high-risk HPV E6 proteins contain at their C termini a PDZ binding domain⁵⁷. A number of various proteins containing the PDZ domain are bound by high-risk HPV E6. This interaction between high-risk HPV E6 and PDZ domain containing proteins lead to targeted degradation of the bound proteins. PDZ motif-containing proteins generally have roles in sustaining signaling transducing multi-protein complexes, as well as preserving intercellular junctions.

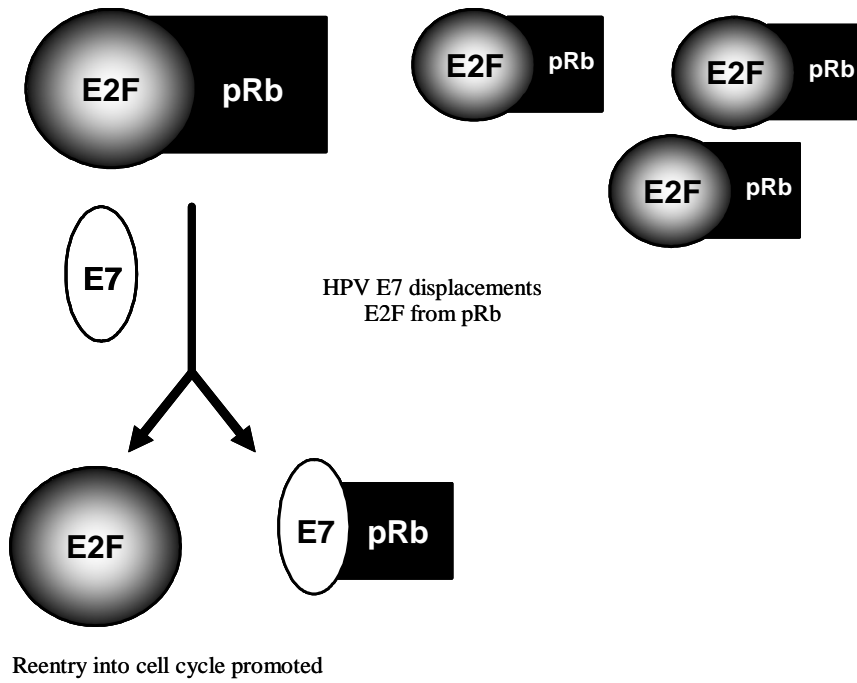
This research seeks to further the field by building upon previous investigations aimed at identifying the multiple transformative properties of the HPV

oncogenic E6 protein. We identified unique properties of the HPV16 E6 protein: the ability to associate with the familial cylindromatosis tumor-suppressor (CYLD), a recognized inhibitor of NF- κ B activation; the ability to inhibit CYLD-mediated suppression of NF- κ B; and the ability to inhibit CYLD-mediated loss of TRAF2. The HPV16 E6 and CYLD association culminates in modulation of CYLD-induced NF- κ B suppression.

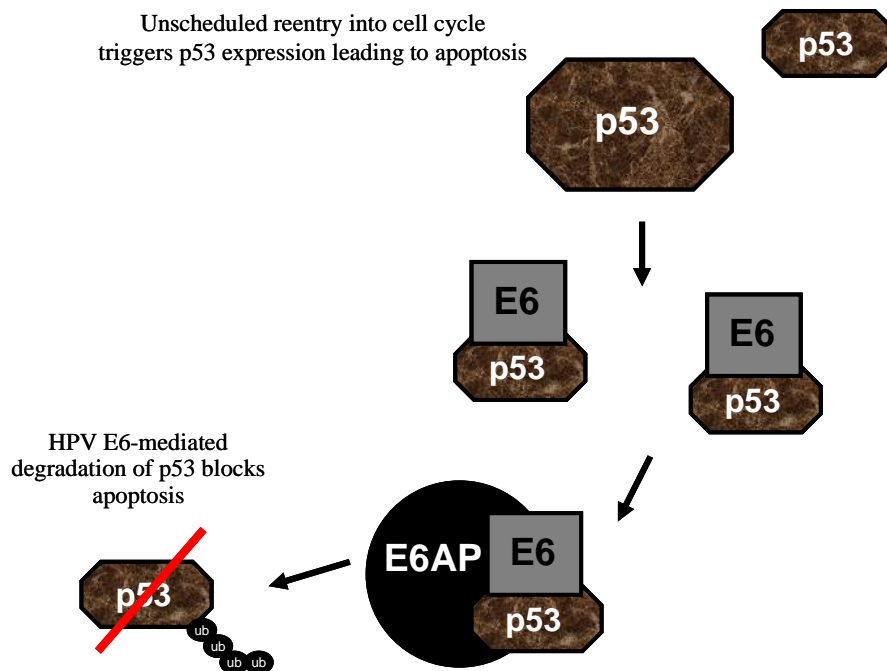
Figure I-4. Classical biochemical actions of HPV oncogenic proteins E7 and E6.

(A) HPV E7 protein binds pRb promoting E2F-mediated cell cycle progression. **(B)** HPV E6 protein binds E6AP and p53, promoting p53-mediated degradation resulting in suppression of apoptosis.

A



B



Summary

The clinical manifestations of human papillomavirus range from the unsightly and bothersome warts to fatal malignancies, most notably cervical cancer. HPV lacks the proteins necessary to complete viral replication independently and must commandeer host cellular proteins to complete its lifecycle. Viral genome integration can occur during the course of persistent HPV infection. Integration frequently triggers continuous expression of viral E7 and E6 proteins in cells, resulting in the disruption of the normal cellular proliferation, cell differentiation and apoptosis. HPV is a very efficient virus, and mediates alterations in a number of normal cellular processes because each of the two oncogenic proteins has multiple responsibilities in HPV-driven tumorigenesis. Over time, with the accumulation of additional mutations, persistent infection of high-risk HPV type can culminate in invasive carcinomas and metastatic cancers.

II. NF- κ B Background

Introduction

HPV E6 expression in keratinocytes promotes nuclear factor κ B (NF- κ B) activation, an observation that complements the known ability of HPV types to induce malignant transformation of cervical epithelium and HPV E6-induced cell immortalization. Though the mechanisms prompting increased NF- κ B activation in the presence of HPV E6 have not been thoroughly investigated, we have shown an association between HPV E6 and a suppressor of the canonical pathway for NF- κ B activation, the cylindromatosis gene product (CYLD), in an effort to elucidate the HPV E6 and NF- κ B relationship

Clinical significance of NF- κ B

Many studies have shown NF- κ B to be a tightly regulated transcription factor, capable of regulating multiple gene products involved in innate immunity, adaptive immunity and inflammation. Additionally, NF- κ B transcriptional activation is associated with up-regulation of genes associated with cellular migration, proliferation and cell survival. Due to the nature of these gene classes, up-regulation of NF- κ B can lead to NF- κ B-mediated alterations of important normal cellular processes, leading to destructive inflammatory states, autoimmune disorders and various other diseases.

NF- κ B-mediated cell proliferation is controlled by activation of target genes such as interleukin-2, granulocyte macrophage stimulating factor and CD40 ligand. Increased transcription of these genes results in protein products that promote growth of predominately myeloid and lymphoid cells. Karin *et al.* demonstrated that the κ B site within cyclin D1 contributes to NF- κ B dependent cyclin D1 induction during pregnancy resulting in mammary epithelial cell growth⁵⁸.

Because NF- κ B pathway dysregulation is associated with increased expression of pro-inflammatory and anti-apoptotic genes, NF- κ B dysregulation has been identified in multiple malignancies. Indeed, NF- κ B is constitutively active in several cancer types, including some gastric cancers, colorectal cancers, breast cancers, leukemias, lymphomas and carcinomas⁵⁹. For example, Hodgkin's lymphoma growth arrest was observed when NF- κ B was blocked⁶⁰. NF- κ B modulates a significant number of genes, resulting in differential expression of genes between normal keratinocytes and transformed or metastatic squamous cell carcinomas⁶¹. Inhibition of NF- κ B in head and neck squamous cell carcinomas decreased tumor proliferation⁶². Additionally, tumor resistance to anti-cancer drugs and ionizing radiation is associated with constitutive expression of NF- κ B.

Many inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and asthma are associated with high levels of NF- κ B activity⁶³. Similarly, in the development of the blood vessels disorder atherosclerosis, invasion of low-density lipoprotein into blood vessel walls is

associated with the activation of NF- κ B and transcription of inflammatory factors promoting fatty plaque formation⁶⁴.

Several viruses and their viral transforming gene products interact with NF- κ B pathway members, and activate NF- κ B-responsive elements. The human T-cell leukemia virus-1 tax protein directly interacts with an inhibitor that normally prevents NF- κ B from moving into the nucleus⁶⁵. Herpes simplex virus-1 activates NF- κ B during early infection and uses NF- κ B to support viral replication^{66,67,68}. Similarly, simian virus 40 large T antigen protein and adenovirus E1A all have been shown to stimulate transcription of NF- κ B responsive genes.

NF- κ B is a family of proteins

NF- κ B, first identified in the laboratory of Nobel laureate David Baltimore, is a protein complex that functions as a primary transcription factor. Five members of the mammalian NF- κ B family currently have been identified. The first class, consisting of RelA (also known as p65), RelB and c-Rel are synthesized as full-length functional proteins (Table II-1). The second class includes NF- κ B1 (sometimes designated as p50 or the precursor p105) and NF- κ B2 (sometimes designated as p52 or the precursor p100). Selective proteolytic processing of c-terminal regions of the p105 and p100 large precursor proteins results in mature p52 and p50 proteins, respectively^{69,70}(Table II-1). The NF- κ B family members all share a well-conserved REL (reticulo endotheliosis) homology domain (RHD) at the

amino terminus. The RHD is a region of about 300 amino acids resembling two immunoglobulin-like domains connected by a flexible linker region. This region is reported to contain the dimerization and nuclear-localization sequence, as well as the DNA-binding and I κ B protein-binding domains. The carboxyl terminal contains ankyrin repeats that mediate protein-protein interactions.

The primary activated complex of NF- κ B that translocates into the nucleus exists as a heterodimer comprised of RelA coupled with p50 or p52. Each NF- κ B family member except RelB can form homodimers. Several heterodimer combinations have also been identified. The processing of NF- κ B1 and NF- κ B2 into p50 and p52 results in the loss of transcription activation ability, so functional p50/p52 heterodimers do not exist.

NF- κ B, maintained in the cytoplasm of cells, is generally noted as being ubiquitously expressed in multiple cell types; however, RelB is believed to be constrained to regions within the thymus, lymph nodes and Peyer's patches, while c-Rel is thought to be limited to lymphoid and hematopoietic cells. DNA binding affinities and ability to activate transcription vary among the different NF- κ B dimer combinations. The RelA and p50 complex exhibit high binding affinity for the DNA consensus 5'-GGGPuNNPyPyCC-3', where Pu is a purine, N is any base, and Py is a pyrimidine^{71,72}.

Table II-1. NF- κ B family members. Class I NF- κ B family members are synthesized in their mature enzymatically active form. Class II NF- κ B family members require proteolytic processing to become the transcriptionally active form.

	Proteolytic processing	Cytoplasmic form	Function
Class I			
RelA (p65)	not required	complexed with I κ B	p65 complexes with p50. This dimer pair represents the classical and most studied NF- κ B protein. p65/p50 NF- κ B is regulated by the canonical pathway.
RelB	not required	complexed with p100	RelB complexes with p52. This dimer pair is activated by the alternative/non-canonical pathway.
c-Rel	not required	complexed with I κ B	Observed in haematopoietic cells and lymphocytes.
Class II			
NF- κ B1 (p105)	yes, into p50	complexed with p65	Functions as a repressor until processed into p50. Lack transcription activation domain.
NF- κ B2 (p100)	yes, into p52	complexed with RelB	Functions as a repressor until processed into p52. Lack transcription activation domain.

NF- κ B pathway signaling

A number of biological triggers have been identified that lead to downstream activation and translocation of the transcription factor NF- κ B, including the bacterial component lipopolysaccharide, lymphotoxin β/α , and exposure to proinflammatory cytokines such as tumor-necrosis factor (TNF)- α , interleukin 1 (IL-1) and CD40 ligand (CD40L). Two unique pathways leading to release of distinct NF- κ B dimers have been noted. The classical, or canonical, pathway leads to release of RelA/p50 heterodimers while the non-canonical pathway leads to the release of predominantly RelB/p52.

Canonical pathway activation is initiated with ligand such as TNF- α and CD40L binding to toll-like receptors (TLR), TNF receptor (TNFR) and T-cell receptors. TNFRs lack enzymatic activity and use TNF-associated factors (TRAFs) directly and indirectly to start signal transduction.

Inactive TRAF proteins are first phosphorylated. Phosphorylation of TRAF proteins mediates TRAF protein ubiquitination. It is predicted that TRAF proteins are lysine 63 poly-ubiquitinated within the ring finger domain of the TRAF proteins⁷³. Ubiquitinated TRAF proteins are active and act upon the downstream protein complex I κ B kinase (IKK). Multiple models have been made as to how the subunits of the IKK complex interact with TRAF proteins to become a functional complex and how TRAF proteins contribute to IKK complex activation.

NF- κ B is sequestered in the cytoplasm by a non-covalent association with I κ B. I κ B bound to NF- κ B veils the nuclear localization sequence positioned within

the RHD of NF- κ B. An exposed nuclear localization sequence is required for NF- κ B shuttling into the cell nucleus. Thus, I κ B serves as a regulatory protein by maintaining NF- κ B in the cytoplasm⁷⁴.

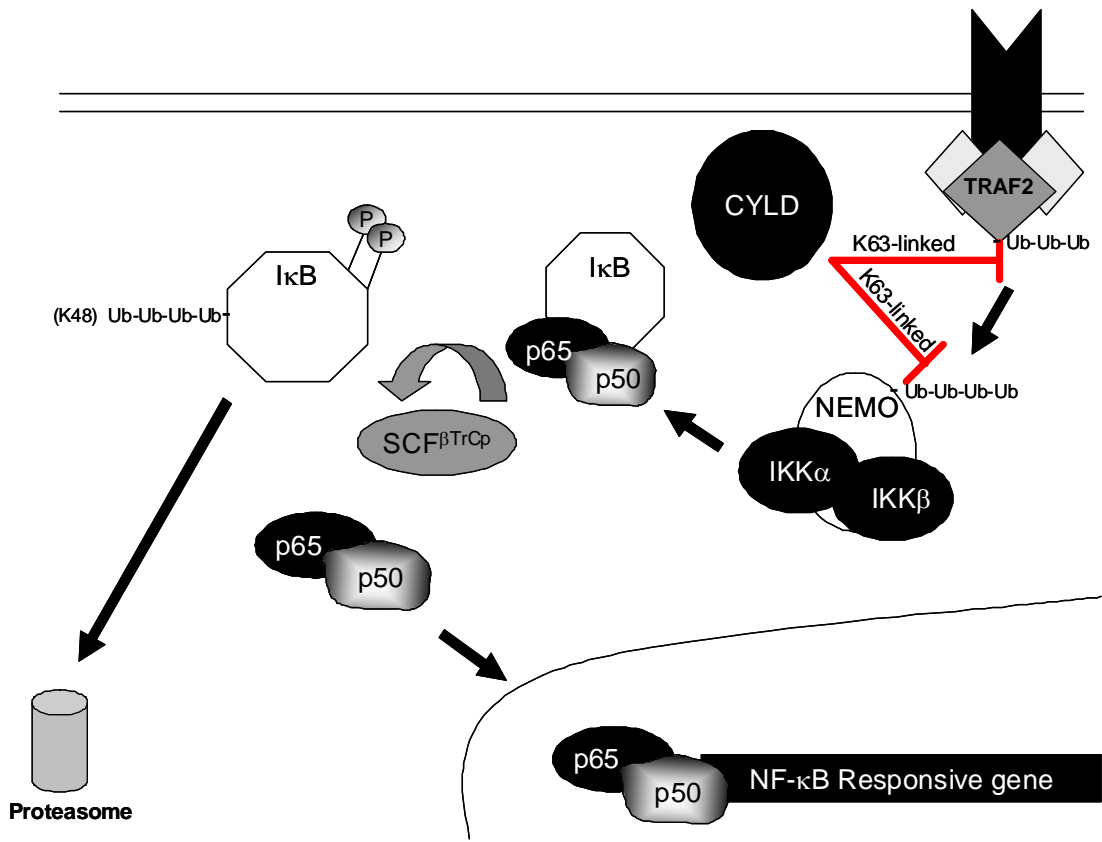
An active IKK complex promotes phosphorylation-induced poly-ubiquitination of I κ B. Briefly, active IKK complex facilitates the phosphorylation of I κ B at serines 32 and 36 in I κ B- α and serines 19 and 23 in I κ B- β ⁷⁵. Phosphorylated I κ B is recognized and poly-ubiquitinated at lysines 21 and 22 by the Skp1-Cullin-F-box (SCF) β -transducin repeat containing protein (β -TRCP) (SCF ^{β TRCP}). SCF ^{β TRCP} is a type E3 ubiquitin-protein ligase that mediates ubiquitination of I κ B leading to its degradation by the 26S proteasome^{76, 77}.

Newly ubiquitinated I κ B releases NF- κ B; the I κ B is degraded. Unbound NF- κ B now has the nuclear-localization sequence exposed. Exposure of the nuclear-localization sequence allows for translocation of NF- κ B into the cell nucleus.

Once inside the nucleus, NF- κ B functions as a transcription factor, and promotes the transcription of a host of genes, especially genes containing a κ B motif. The κ B motif is a nine to ten base pair DNA sequence recognized by NF- κ B.

Figure II-1. Classical NF- κ B pathway. Depiction of the NF- κ B classical pathway.

CYLD is shown to suppress the pathway by deubiquitinating TRAF2 and NEMO.



NF- κ B pathway associated proteins

TRAF proteins

TNFR family members use TRAF proteins as adaptor proteins for signal transduction. Six TRAF family members have been characterized. TRAF1 lacks the ring finger domain characteristic of the other family members. The ring finger domain assists in the addition of lysine 63-linked poly-ubiquitin chains. Essentially, TRAF proteins function as scaffolding proteins to help assemble downstream complexes required for NF- κ B activation. For example, through interactions with the IKK- α and IKK- β subunits of the IKK complex, TRAF2 recruits the IKK complex to the activated TNFR1⁷⁸.

IKK complex

The IKK protein is a complex of three subunits, IKK- α , IKK- β and IKK- γ (NEMO). TRAF protein activation is sufficient for IKK activation; however, a detailed picture of this phenomenon is under investigation. Interestingly, the regulatory subunit NEMO has been shown to be vital for functional activity of the IKK complex, triggering further NF- κ B pathway signaling. Although NEMO is essential for IKK complex activity; the mechanism of IKK complex activation is not completely understood. Three current hypotheses for IKK activation have been postulated. First, IKK subunits are assembled and the IKK complex becomes active following phosphorylation by upstream IKK kinase. The second hypothesis suggests that toll-like receptors direct autophosphorylation or a transautophosphorylation

subunit interplay between NEMO and the other two subunits of the IKK complex, IKK- α /IKK- β ⁷⁷ resulting from NEMO oligomerization^{79, 80}. The last prediction is that NEMO functions to direct translocation of the IKK complex subunits to currently unidentified proteins that mediated IKK complex formation and phosphorylation.

Overall, IKK complex activation is dependent upon NEMO. Another function of NEMO is NEMO's involvement in NF- κ B activation in response to DNA damage. Sumoylation of NEMO leads to NEMO translocation into the nucleus where the ataxia telangiectasia mutated kinase leads to NEMO ubiquitination⁷⁷. Upon ubiquitination, NEMO moves back into the cytoplasm to interact with IKK- α and IKK- β subunits resulting in an active IKK complex.

The presence of the IKK- β subunit of the IKK complex is essential for efficient IKK complex activity in the canonical pathway; the absence the IKK- β subunit in a cell system showed decreased I κ B degradation⁶³. Halting I κ B degradation allows more I κ B to be available to sequester NF- κ B. Bound NF- κ B does not translocate into the nucleus.

IKK- α is essential for NF- κ B alternative (non-canonical) pathway activation. The IKK- α subunit of the IKK complex assists in the proteolytic process cleaving NF- κ B2 (also known as p100) into the p52 protein that makes up the NF- κ B heterodimer used in the NF- κ B alternative pathway^{81, 63}(Table II-1). The IKK- α subunit has a role in induction of keratinocyte differentiation⁸¹. Additionally IKK-

α forms a complex with NF- κ B and other transcription initiation factors on the promoter sequence of NF- κ B responsive genes⁸².

I κ B

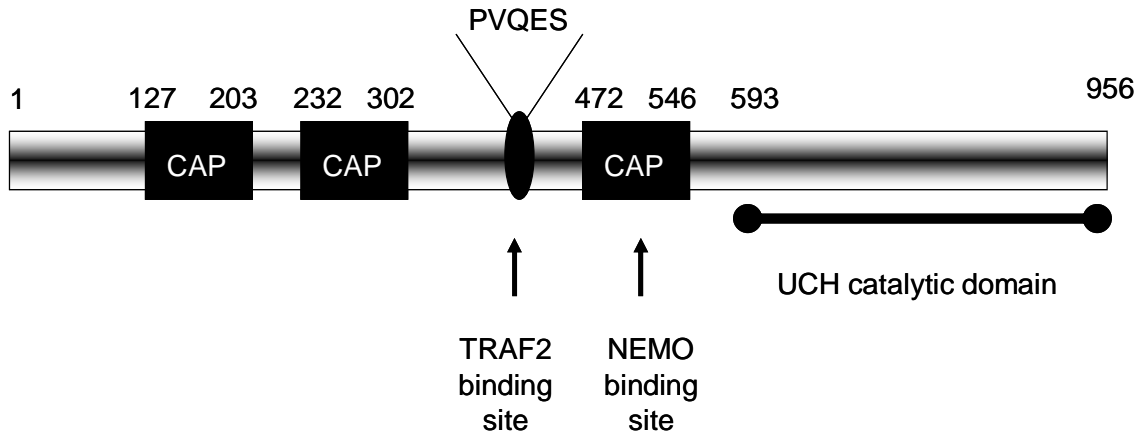
I κ B binds NF- κ B. This I κ B/NF- κ B complex maintains NF- κ B in the cytoplasm by masking the nuclear localization signal harbored by NF- κ B. The I κ B family members currently recognized includes I κ B- α , I κ B- β , I κ B- ε and closely related Bcl-3.

CYLD

Mutations in the cylindromatosis tumor suppressor gene (CYLD gene) have resulted in the formation of often benign tumors of skin appendage called cylindromas. The CYLD protein is made up of 956 amino acids and has three cytoskeleton-associated protein-glycine conserved domains (CAP-Gly) near the amino terminal and an ubiquitin carboxyl-terminal hydrolase catalytic domain (UCH)⁸³ (Figure II-2). The UCH motif within CYLD is similar to cysteine and histidine boxes found in the carboxyl terminus of ubiquitin-specific protease subfamily of enzymes with deubiquitination activity⁸⁴. The third CAP-Gly domain and the UCH domain are essential for binding TRAF2, NEMO⁸⁵ and Bcl-3⁸⁶. CYLD is a deubiquitinase; as ubiquitinated TRAF2, NEMO, and Bcl-3 have been identified as substrates for the CYLD ubiquitin protease activity. CYLD removes stimulatory lysine 63-linked ubiquitin chains, instead of lysine 48-linked chains that are known

to lead to proteasome directed degradation of proteins. The lysine 63-linked ubiquitin chain is involved in TRAF2 and NEMO activation of the NF- κ B pathway. CYLD is identified as an endogenous inhibitor of NF- κ B activation; a mechanism behind this observation is CYLD deubiquitination of TRAF2 and NEMO. Further studies confirmed a role for CYLD in NF- κ B pathway activation by showing that silencing of CYLD expression increased NF- κ B activation, both at baseline, and especially in cytokine TNF- α and CD40L stimulated cells^{87,88}. Both a CYLD deficiency and suppression of CYLD expression in cultured cells increases apoptosis resistance, possibly resulting in oncogenesis.

Figure II-2. Schematic representation of CYLD protein. The CYLD protein has three cytoskeleton-associated protein-glycine conserved domains (CAP) near the amino terminus and an ubiquitin carboxyl-terminus hydrolase catalytic domain (UCH). The TRAF2 and NEMO binding sites are indicated by arrows. The sequence PVQES is required for TRAF2 binding.



Summary

NF- κ B is a family of proteins with transcriptional activity. NF- κ B proteins are normally sequestered in the cellular cytoplasm by I κ B regulatory proteins. Upon stimulation by either a variety of cytokines or through pathogen infection, a cascade of biochemical events occurs resulting in the involvement of the IKK complex subunits IKK- α , IKK- β and NEMO. IKK complex subunits trigger the release of sequestered NF- κ B by I κ B, unveiling the nuclear localization sequence of NF- κ B, resulting in NF- κ B shuttling into the nucleus. Once in the nucleus, NF- κ B is able to promote transcription of tightly regulated genes involved in immune and inflammatory responses, cell migration, apoptosis inhibition, cellular proliferation and angiogenesis. Transcription of NF- κ B responsive genes alters normal cell physiology and contributes to cellular changes associated with inflammatory disorders and oncogenesis.

III. The HPV E6 protein binds CYLD and modulates NF- κ B

Introduction

HPV infection of primary keratinocytes is characterized by activation of the NF- κ B pathway, resulting in an anti-apoptotic state contributing to cell survival and cellular proliferation^{89, 90, 91}. Nees *et al.* reports that infection of keratinocytes with a retrovirus construct encoding the HPV16 E6 protein enhanced gene expression of multiple functional components of the NF- κ B signaling pathway, including tumor necrosis factor (TNF)-receptor associated factor-interacting protein (TRIP), NF- κ B-inducing kinase (NIK), RelA (a NF- κ B family member comprising the NF- κ B dimer after proinflammatory cytokine exposure induction or response to viral infection); and induced NF- κ B binding to its DNA consensus sites⁵⁶. These results support the observation that activation of NF- κ B within HPV-infected cells is stimulated by the E6 protein. James *et al.* established a role for HPV E6 in up-regulating p52 NF- κ B containing dimers using a NF- κ B responsive luciferase report assay⁹².

In an effort to identify how HPV E6 expression leads to NF- κ B activation, we have found that HPV E6 binds to the cylindromatosis tumor suppressor protein, CYLD. A CYLD protein deficiency results in familial cylindromatosis, an autosomal dominant genetic predisposition to multiple neoplasms, or cylindromas, that are believed to arise from skin appendages such as the eccrine (sweat) and apocrine

(scent) cells of the skin^{83,93,94}. CYLD deficiency in epithelial cells leads to development of cylindromatosis and cylindroma formation⁸³.

The carboxyl terminus of CYLD contains a deubiquitinating domain that removes lysine 63 linked polyubiquitin chains from TNF-receptor associated factor 2 and 6 (TRAF2 and TRAF6) (Fig. II-2). K63 deubiquitination of TRAF2 inhibits TRAF2-mediated activation of the IKK complex and ultimately prevents the dissociation and translocation of NF- κ B^{87,95,88}. It is also proposed that CYLD can directly bind to the regulatory subunit of the IKK complex, NEMO, again resulting in inhibition of NF- κ B activation^{87,88,96,95}. Through these actions, CYLD functions as a suppressor of NF- κ B activation^{95,96}. Brummelkamp *et al.* have shown that silencing of CYLD expression in cultured cells leads to increased NF- κ B activation, both at baseline, and especially following cytokine stimulation⁸⁷.

Given the ability of HPV E6 to activate NF- κ B, and the central role of the CYLD protein in suppression of the canonical pathway for NF- κ B activation, we hypothesized that HPV E6 proteins block established CYLD biochemical activities. In this study, we observed that HPV E6 proteins bind CYLD, and showed HPV16 E6 abated CYLD-mediated NF- κ B suppression.

NF- κ B activation results in the transcription of a variety of genes involved in proliferation, immune and inflammation responses, and apoptosis resistance (for reviews, see^{97,98}); which are all contributory effects in carcinogenesis. Furthermore, chronic inflammation is an important factor in carcinogenesis^{99,100,101}, particularly among epithelial tumors such as cervical cancers¹⁰² (reviewed in⁴⁰). Thus, we

propose that HPV E6 contributes of HPV-induced carcinogenesis by inducing a cellular environment stimulating NF- κ B translocation to the nucleus for transcription of proliferative and anti-apoptotic genes (canonical pathway activation).

Methods

Cell Lines and Tissue Culture.

HPV negative cell lines: Human embryonic kidney cell line 293T/17 (293T cells), human prostate cancer cells (PC3) and cervical cancer cell line C33-A (C33-A). 293T cells were grown in HyQ Dulbecco's MEM with 4 mM L-glutamine, 4.5 g/L glucose and sodium pyruvate (HyClone). PC3 cells were grown in RPMI 1640 supplemented with 2 mM L-glutamine and penicillin (100 IU/mL). C33-A cells were grown in MEM (HyClone) with 2 mM L-glutamine. H1299 human lung adenocarcinoma cells were cultured in RPMI 1640.

HPV positive cell lines: HeLa (a cervical cancer cell line transformed with HPV-18), SiHa cells (a cervical cancer cell line transformed with HPV-16), and W12-E cells (a cervical cell line that maintains extrachromosomal HPV-16). HeLa and SiHa cells were grown in MEM. W12-E cells were grown on mitomycin C (4 μ g/mL) treated 3T3 mouse fibroblasts in F-medium supplemented with hydrocortisone (0.4 μ g/mL), cholera toxin (0.1 nM), insulin (5 μ g/mL), adenine (25 μ g/mL), epidermal growth factor (10 ng/mL) and 5% FBS, as previously described^{103,104}. All other cells were cultured in media supplemented with 10% fetal bovine serum.

Dual reporter luciferase assays

293T cells seeded in 24 well tissue culture plates were transfected with 1 ng of pRL-tk (an internal transfection efficiency control plasmid that expresses *Renilla* luciferase under control of an SV40 promoter), 0.3 µg pNF-κB-Luc (a plasmid that expresses firefly luciferase under control of an NF-κB responsive promoter) and 0.2 µg of pCD40L (a plasmid that constitutively expresses CD40 ligand, provided by Dr. George Mosialos, Aristotle University of Thessaloniki, Greece) (Fig. III-1a). Simultaneously, the cells were co-transfected with either 0.2 µg of plasmid expressing HPV-11 E6, 0.2 µg of plasmid expressing HPV-16 E6, 0.3µg of plasmid expressing CYLD or a combination of HPV-11 E6 and CYLD plasmids or HPV-16 E6 and CYLD plasmids. After 24 hours, transfected cells were harvested in 1x passive lysis buffer (Promega). Firefly luciferase and *Renilla* luciferase enzymatic activity were measured consecutively according to the dual-luciferase reporter assay protocol (Promega) using 10 µL of lysate in a 20/20ⁿ luminometer (Turner BioSystems). Final DNA concentrations for all transfections were held constant by using empty control vectors. Each experiment was performed in triplicate and analyzed as an arithmetic mean. Statistical significance of data presented were calculated by *t* test analysis, with P<0.05 being considered significant.

In the dual luciferase assay examining p53 independence, p53 null H1299 cells (CRL-5803, ATTC) were used instead of 293T cells. The assay was completed as described previously (Fig. III-2a). In the dual luciferase assay examining p53

independence using HPV16 E6 mutant F2V, 293T cells were transfected with an NF- κ B-responsive element-luciferase fusion plasmid, with a *Renilla* luciferase control plasmid, and with plasmids expressing CD40L, CYLD, and F2V. The assay was completed as described previously (Fig. III-2b)

Immuno precipitation assays

To determine if the HPV E6 protein binds CYLD, HPV11 and HPV16 E6-flag fusion proteins and HA labeled CYLD were independently expressed in 293T cells. Cells were harvested and lysed. CYLD containing lysate was mixed with either HPV16 E6 or HPV11 E6 containing lysates. As a control, HPV11 and 16 E6 were mixed with lysate from cells transfected with empty flag vector. Following incubation, the proteins in the mixtures were precipitated using anti-FLAG M2 agarose (Sigma). The resin was washed thoroughly, proteins were eluted with SDS buffer and fractionated by SDS-PAGE. The presence of the CYLD protein was detected using anti-CYLD antibody (Orbigen). The reverse experiment was also completed when HPV11 and HPV16 E6-HA fusion proteins and flag labeled CYLD were expressed in 293T cells. As stated above, cells were harvested, lysed and the CYLD and E6 lysates were mixed. Following incubation the proteins in the mixture were precipitated using anti-FLAG M2 agarose (Sigma), thoroughly washed, and HPV16 E6 and HPV11 E6 were detected using anti-HA antibody. HPV11 E6 and HPV16 E6 were both detected to bind CYLD.

RESULTS

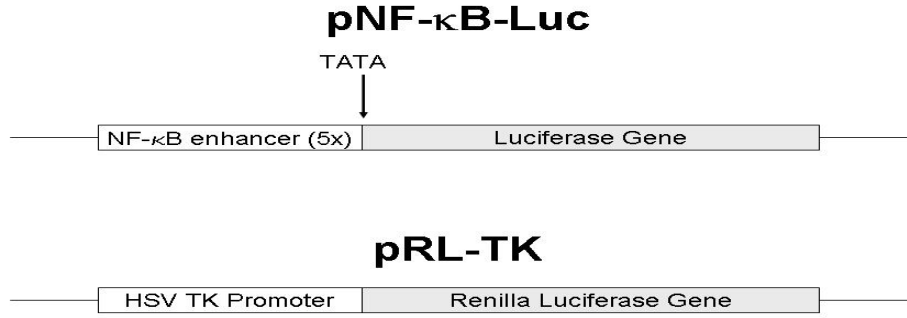
HPV16 E6 antagonizes the ability of CYLD to suppress NF- κ B activation

HPV E6 protein expression in cultured cells leads to NF- κ B activation^{89,90}.¹⁰⁵ To guide our studies of this phenomenon, we hypothesized that expression of HPV E6 proteins would inhibit the ability of CYLD to suppress NF- κ B pathway activation. To measure changes in NF- κ B activation, we used a dual luciferase reporter assay system (See Fig. III-1a for vector constructs). Nuclear NF- κ B trans-activation activity was quantified in cultured cells as a function of HPV E6 and CYLD expression. 293T cells were transiently transfected with a NF- κ B-luciferase reporter construct, a *Renilla* luciferase internal control and with plasmids expressing HPV E6 proteins and CYLD. Cells not stimulated with CD40 ligand (CD40L) expressed little NF- κ B; CD40L stimulation resulted in high levels of NF- κ B expression, resulting in a strong luciferase mediated luminescence (Fig. III-1b, lane 2). Expression of CYLD in CD40L-stimulated cells inhibited the marked up-regulation of NF- κ B activity seen in the presence of CD40L alone. Expression of HPV11 E6 or HPV16 E6 in CD40L-treated cells resulted in NF- κ B activity comparable to the NF- κ B activity in cells treated with CD40L alone (Fig. III-1b, lanes 4 and 6). In cells co-transfected with HPV16 E6 and CYLD, we found that the introduction of HPV16 E6 reversed the CYLD-mediated inhibition of CD40L-

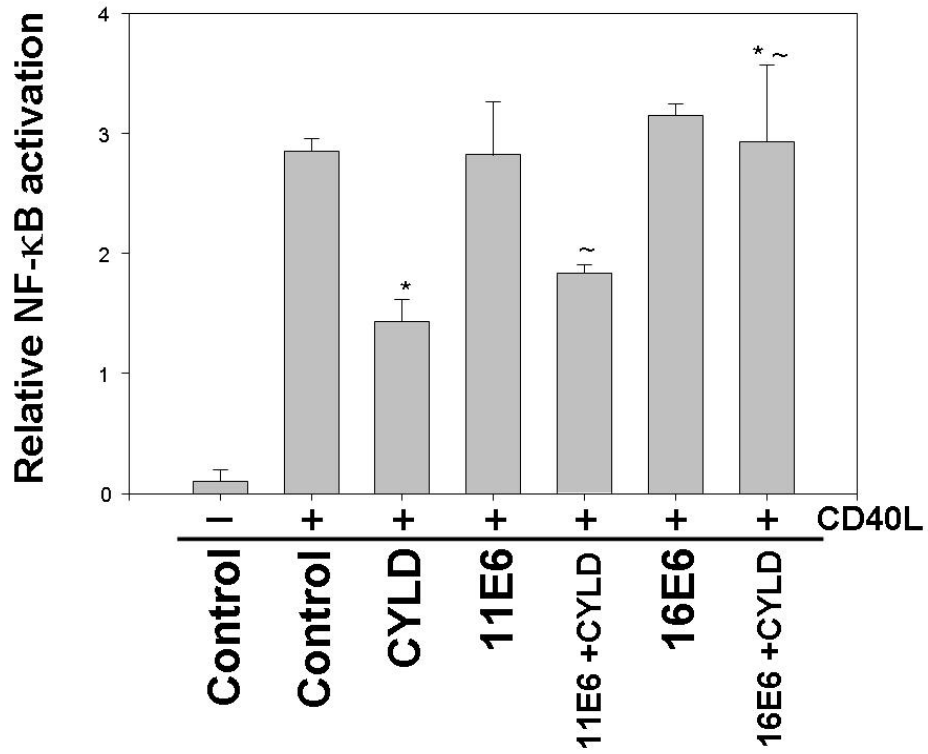
induced NF- κ B activation observed with CYLD alone. In contrast to our findings with HPV16 E6, we found that the expression of HPV11 E6 did not fully reverse the NF- κ B inhibiting effects of CYLD (compare Fig. III-1b, lanes 3-7).

FIGURE III-1. Characterization of HPV16 E6 and HPV11 E6 effects on NF- κ B activation. (A) NF- κ B-responsive element-luciferase fusion plasmid and *Renilla* luciferase control plasmid. (B) HPV16 E6 blocks the ability of CYLD to suppress NF- κ B activation. 293T cells were transfected with an NF- κ B-responsive element-luciferase fusion plasmid, with a *Renilla* luciferase control plasmid, and with plasmids expressing CD40L, CYLD, HPV11 E6, and HPV16 E6 as indicated. *, P<0.02; ~, P<0.05. P values determined by *t* test analysis.

A



B



E6-mediated NF- κ B activation does not require p53 tumor suppressor protein

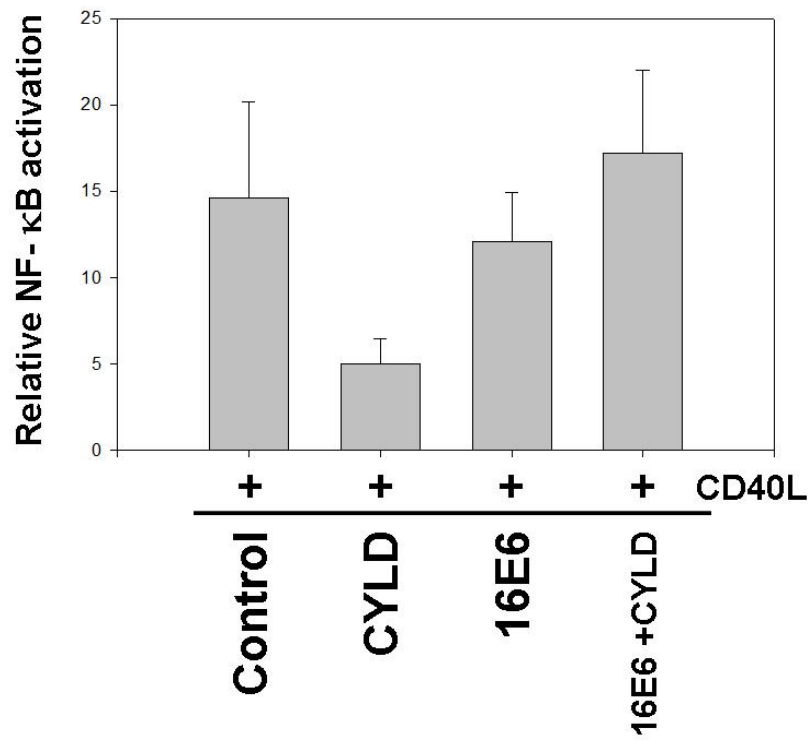
Because a well described activity of high-risk E6 proteins is promotion of p53 degradation, we hypothesized that the effects of HPV16 on the canonic NF- κ B pathway were independent of the ability of high-risk E6 proteins to drive p53 degradation. To confirm if the observed HPV16 E6-mediated activation of NF- κ B was p53 independent, the ability of the HPV16 E6 to attenuate CYLD-mediated suppression of NF- κ B activation in the p53 negative cell line H1299 was assayed using the dual luciferase reporter assay system previously described. The ability of HPV16 E6 to inhibit CYLD-mediated suppression of NF- κ B activation was recapitulated in this p53 null cell line, thereby suggesting that the CYLD-inhibiting activity of HPV16 E6 is independent of p53 degradation.

As a second independent method, we used a HPV16 E6 mutant F2V in our dual luciferase assay system as described above. F2V has been previously characterized as having the ability to bind p53 but lacking the ability to promote E6- and E6AP-mediated proteasomal degradation of p53. Expression of CD40L resulted in an increase in relative NF- κ B that was abated by expression of CYLD (Fig. III-2b). Expression of F2V increased NF- κ B levels relative to CYLD alone; however, F2V expressed alone was not statistically different from the control. F2V, the HPV 16 E6 mutant lacking p53 degradation ability, reversed CYLD-mediated suppression of NF- κ B (Fig. III-2b, compare lanes 3 and 5). This further confirmed the

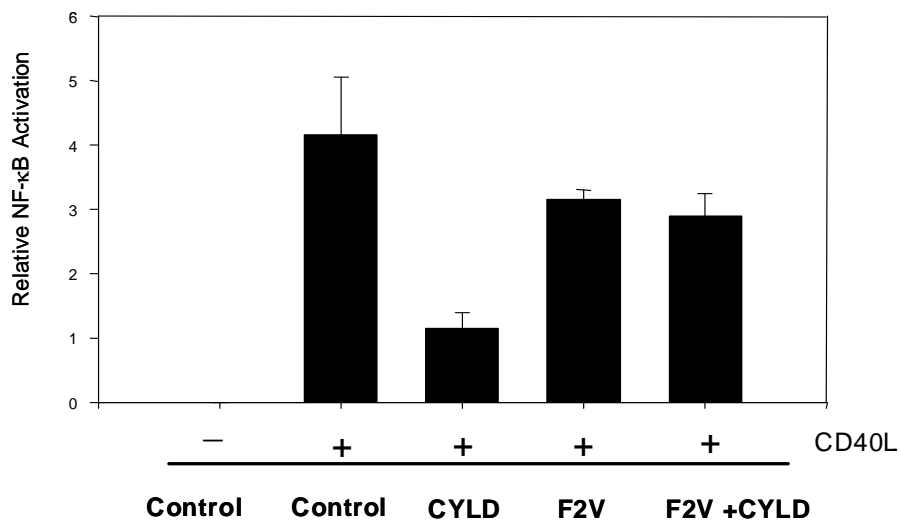
observation that HPV16 E6-mediated activation of NF- κ B in the presence of CYLD appears to be a p53 independent role of HPV16 E6.

Figure III-2. HPV16 E6 blocks the ability of CYLD to suppress NF- κ B activation in cells deficient in p53. (A) p53 null H1299 cells (CRL-5803, ATTC) were transfected with an NF- κ B-responsive element-luciferase fusion plasmid, with a *Renilla* luciferase control plasmid, and with plasmids expressing CD40L, CYLD, and HPV16 E6. Comparing lanes 2 and 4, $P < 0.02$. P values determined by *t* test analysis. (B) HPV E6 F2V mutant lacking p53 degradation ability was used in a luciferase assay. Results shown are the mean \pm s.d. relative luciferase activity from three independent samples.

A



B

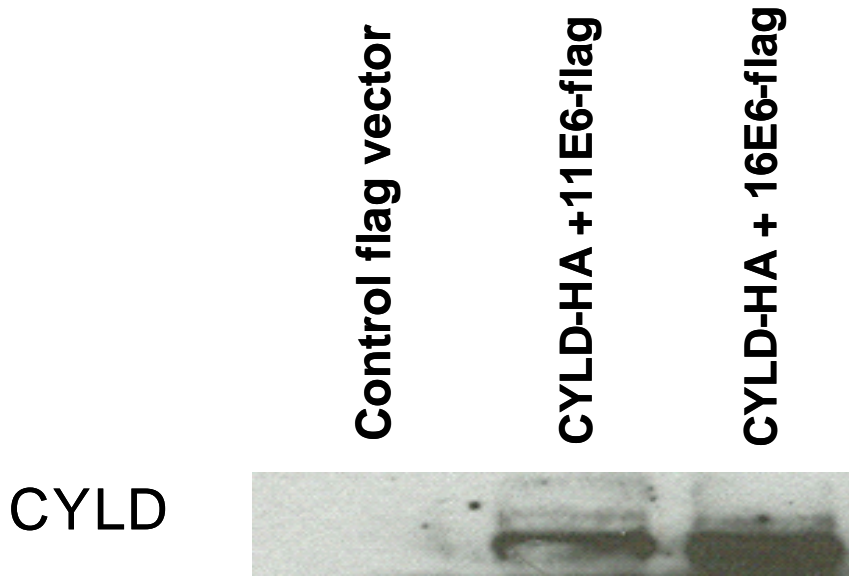


HPV E6 proteins bind CYLD

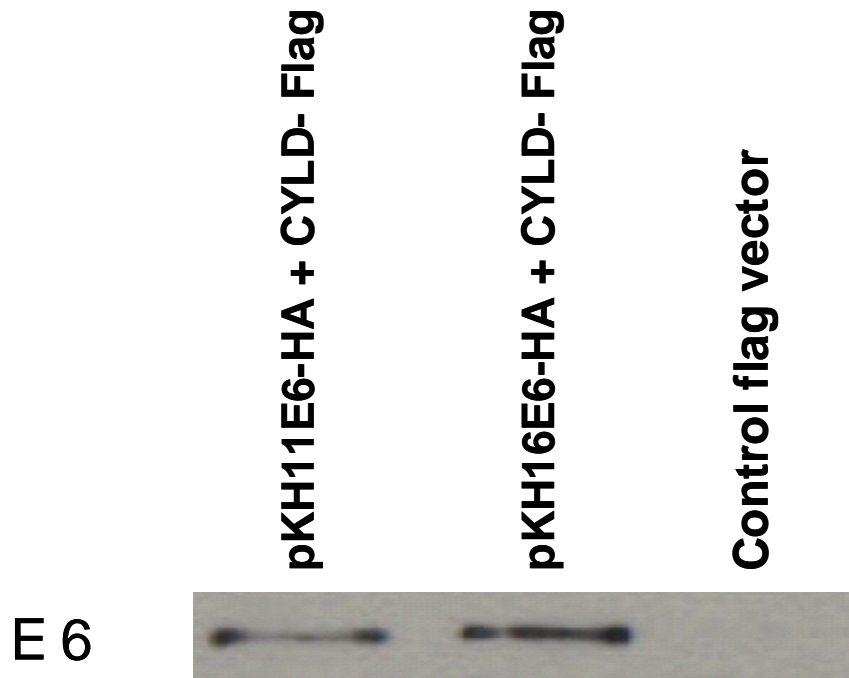
Given the ability of HPV16 E6 to reverse CYLD-mediated inhibition of NF- κ B activation, we hypothesized that the effect of HPV16 E6 on NF- κ B activation was mediated through direct E6:CYLD interaction. To determine if E6 bound CYLD, E6 and CYLD co-immunoprecipitation experiments were performed. Surprisingly, we found that both HPV E6 types 11 and 16 proteins co-precipitated the CYLD protein (Fig. III-3a). The reverse experiment was performed and CYLD co-precipitated E6 proteins (Fig. III-3b). The ability of the HPV16 E6 proteins to co-precipitate CYLD is consistent with the hypothesis that the E6:CYLD interaction *in vivo* is essential to the molecular basis of E6-mediated NF- κ B pathway activation.

Figure III-3. The HPV E6 protein binds CYLD. (A) HPV11 and HPV16 E6-flag fusion proteins and HA labeled CYLD were expressed in 293T cells. Cell lysates were used in a co-immunoprecipitation assay. CYLD-HA was detected using anti-CYLD antibody. (B) HPV11 and HPV16 E6-HA fusion proteins and flag labeled CYLD were expressed in 293T cells. E6-HA was detected using anti-HA antibody. HPV11 E6 and HPV16 E6 were both detected to bind CYLD.

A



B



DISCUSSION

HPV has been shown to activate the NF- κ B pathway. In an effort to identify how HPV expression leads to NF- κ B pathway activation, we have found that the HPV16 E6 protein interacts with the deubiquitinase CYLD. A well studied form of posttranslational protein management involves the addition of ubiquitin. In the process of ubiquitination, ubiquitin is covalently attached to target proteins in an extremely specific process, commonly as poly-ubiquitin chains, via the lysine residues of a protein^{106,107}. Lysine 63-linked ubiquitin modification activates TRAF proteins, promoting IKK complex formation and stimulation (for review see Chapter II). Many researchers are examining the role of ubiquitin modulation in relation to viruses and have identified viral proteins that have ubiquitin ligase activity including the MIR1 and MIR2 proteins of human herpesvirus 8¹⁰⁸. There are also viral proteins that interact with ubiquitin ligases and proteases; such as adenovirus proteins, E4 34k and E1b 55k. E4 34k and E1b 55k form complexes to participate in a virus-specific E3 ubiquitin ligase-mediated ubiquitination and proteasomal degradation of Mre11¹⁰⁹, p53¹¹⁰ and DNA ligase IV¹¹¹.

Finding an interaction among HPV and known ubiquitin altering enzymes could define alternate oncogenic mechanisms used by HPV. We explored the possibility of HPV proteins interacting with a recently characterized protein, CYLD. The loss of CYLD is known to alter epithelial derived cells, resulting in benign neoplasms reminiscent of the growths caused by HPV.

For our studies, a combination of vectors expressing CYLD and either HPV16 E6 or HPV11 E6 were transfected into 293T cells. A plasmid encoding CD40L was also added to activate the canonical NF- κ B pathway. NF- κ B pathway activation was then measured using an NF- κ B-responsive dual luciferase reporter system. In keeping with the findings of others, we found that CYLD was a potent suppressor of CD40L-mediated NF- κ B pathway activation^{87,88,95}.

We also found that, while expression of HPV16 E6 or HPV11 E6 alone in 293T cells did not significantly alter NF- κ B levels (Fig. III-1b, lanes 4 and 6), co-expression of HPV16 E6 with CYLD in CD40L-stimulated cells attenuated the characteristic CYLD-mediated suppression of NF- κ B activation (Fig. III-1b, lane 7). This attenuation of CYLD activity by HPV-16 E6 was not seen with HPV-11 E6. These results indicate that HPV16 E6 antagonizes the NF- κ B pathway-suppressing activity of CYLD, leading to persistent NF- κ B activation. Because NF- κ B expression in keratinocytes confers apoptosis resistance¹¹², we propose that the ability of HPV16 E6 to inhibit CYLD-mediated suppression of the canonical pathway of NF- κ B activation contributes to the proliferative phenotype of HPV-infected and HPV-transformed cells. This HPV16 E6:CYLD interaction and consequent inhibition of CYLD activity constitutes a novel mechanism by which the HPV16 E6 protein could induce an anti-apoptotic cellular state in HPV16 infected and HPV16 transformed keratinocytes.

In this study, we hypothesized that the NF- κ B-activating activity of HPV16 E6 was not mediated through direct HPV E6:p53 interaction. To test this hypothesis,

we measured the ability of the HPV16 E6 to block CYLD-mediated NF- κ B pathway suppression in the p53-deficient cell line H1299. In H1299 cells, HPV16 E6 maintained the capacity to reverse CYLD-mediated inhibition of NF- κ B activation (Fig III-2).

We further hypothesized that the effects of HPV E6 on CYLD could be mediated through direct HPV E6:CYLD association. Using standard co-precipitation methods, low-risk HPV type 11 proteins E2, E6 and E7 as well as high-risk type 16 proteins E6 and E7 were assayed (data not shown). Only the HPV E6 proteins of both high- and low-risk types readily co-precipitated CYLD in our system (Fig. III-3). Interestingly, HPV11 E6 was able to bind CYLD but did not show ability to inhibit CYLD suppression of NF- κ B activation. The significance of the HPV11 E6 and CYLD association needs further study.

The significance of the HPV E6 and NF- κ B interaction arises from the anti-apoptotic effects of NF- κ B. Activation of NF- κ B results in transcription of a variety of genes involved in keratinocyte proliferation, immune and inflammation responses, and protection against apoptosis^{98,97}, all factors that could contribute to malignant conversion of keratinocytes. The persistent NF- κ B activation induced by HPV E6 has the hallmarks of chronic inflammation. Chronic inflammation is an important factor in carcinogenesis^{99,100,101}. Prominent among inflammation-induced malignancies are epithelial tumors¹⁰². Therefore, chronic inflammation may contribute to the development of cervical cancers¹⁰¹.

It is possible that the HPV16 E6 protein may affect other steps in the pathway leading to NF- κ B activation. It has been determined previously that both the canonical and non-canonical NF- κ B pathways can be activated by CD40L^{113, 114, 115}; thus, our data do not exclude a role for HPV E6 in the modulation of an alternate pathway for NF- κ B activation.

The HPV E6 protein is a central factor in HPV-induced cellular transformation and in the development of cervical dysplasia and cervical cancer^{116,91,117}. The HPV E6 protein prevents apoptosis in several ways, thereby contributing to the proliferative and malignant phenotype of HPV-infected and HPV-transformed cells. In this study, we have shown the HPV16 E6:CYLD is sufficient to abate CYLD-mediated inhibition of NF- κ B and may be paramount in leading to NF- κ B activation by HPV.

Summary

HPV infection of primary keratinocytes leads to activation of the NF- κ B pathway, promoting an anti-apoptotic state. This activation of NF- κ B is stimulated, at least in part, by the HPV E6 oncoprotein. We proposed that HPV E6 protein promotes NF- κ B pathway activation by interfering with the activity of CYLD, an important regulator of NF- κ B activation.

We have shown that HPV E6 proteins associated with CYLD *in vitro*. High-risk HPV16 E6 and not low-risk HPV11 E6 expression in 293T cells blocks the ability of CYLD to inhibit CD40 ligand-stimulated NF- κ B activation.

IV. HPV16 E6 rescues CYLD-mediated TRAF2 loss

Introduction

A major contribution to our understanding of the role of CYLD as a tumor suppressor, and as a deubiquitinase occurred when several labs reported the ability of CYLD to reverse K63-linked ubiquitination of TRAF2 and NEMO. CYLD mediated deubiquitination of TRAF2 and NEMO results in attenuation of canonical NF- κ B pathway activation. TRAF2 interacts with the cytoplasmic domain of TNFRs, serving as an adaptor protein by recruiting proteins to the site to enhance intracellular signal transduction^{118,119}. We determined that expression of CYLD induced loss of TRAF2, and that CYLD-mediated loss of TRAF2 could be abated by co-expression of CYLD with HPV16 E6.

Our data showing CYLD expression resulted in TRAF2 loss and the data showing HPV16E6 can prevent CYLD-mediated TRAF2 loss was completed by over-expressing TRAF2, CYLD and HPV16E6. Over-expression experiments should be validated using other research methods. To provide an alternative to the over-expression data we determined the effect of introducing siRNA against HPV16 E6 in a cell line transformed with HPV16. We observed silencing of HPV16 E6 resulted in a loss of endogenous TRAF2.

It has been demonstrated that endogenous CYLD undergoes NEMO dependent phosphorylation and that this CYLD phosphorylation was essential for

regulation of signal induced ubiquitination of TRAF2^{120, 95}. TRAF2 was constitutively K63 ubiquitinated in the absence of CYLD. Reiley *et al.* proposed that phosphorylation of CYLD results in deactivation of CYLD¹²⁰. Given the ability of HPV E6 to bind CYLD, we wanted to examine the functional consequences of this interaction and examine CYLD phosphorylation in the presence of HPV E6. We determined HPV E6 increased CYLD phosphorylation. HPV E6 mediated phosphorylation of CYLD may serve as a mechanism for the HPV16 E6 abatement of CYLD suppression of NF-κB activation (reviewed in Chapter III).

Methods

Western blot analysis for measurement of TRAF2 levels

Protein levels of interest were assayed using established western blot techniques. Briefly, 293T cells at 60-70% confluency in 12 well plates were transfected using FuGENE 6 Transfection Reagent (Roche) according to manufacture provided instructions. Cells were transfected with 0.3 μg of flag-tagged plasmids pcDNA-TRAF2-flag (TRAF2) and pcDNA3-CYLD-flag (CYLD). PKH3-16E6-HA or pKH3-11E6-HA influenza hemagglutinin E6 fusion plasmids were simultaneously transfected. All transfections were repeated in duplicate and cells were incubated for 24-48 hours. Cells were harvested in 1x lysis buffer (40 mM Tris-HCl pH 7.6, 150 mM NaCl, 1mM PMSF, 1% NP-40) and the lysate was resolved in gradient SDS-PAGE gels before being transferred to nitro-cellulose membrane on a

trans-blot SD semi-dry electrophoretic transfer cell (Bio-Rad). TRAF2 protein was detected using an anti-flag M2 peroxidase conjugate (Sigma) or anti-TRAF2 (H249) antibody (Santa Cruz Biotechnology). Immunoblots were developed with ECL-pico chemiluminescent reagent (Pierce).

Suppression of E6/E7 expression with an anti-E6/E7 siRNA

A 19-nucleotide duplex siRNA homologous to bases 142-160 of the HPV 16 E6/E7 open reading frame with 2-nucleotide deoxythymidine 3' overhangs was used to reduce endogenous E6/E7 expression¹²¹. Anti-HPV 16 E6/E7 or control siRNA was transfected into subconfluent cells using oligofectamine reagent (Invitrogen) and Opti-MEM (reduced serum medium (Gibco)) according to the manufacturer's protocol. The cells were harvested 48 hours later in RIPA buffer (40 mM Tris-HCl pH, 150 mM NaCl, 1mM PMSF, 1% NP40). Protein expression was analyzed by western blot.

CYLD phosphorylation assay

293T cells were transfected with either HPV11 E6, HPV16 E6 or empty vector. Eighteen hours after transfection, 2 mCi of [³²P] orthophosphate, chemical form H₃PO₄ in H₂O (MP Biomedicals), was added to the cell medium for 2-4 hours. Cell lysates were mixed with anti-CYLD antibody and protein A-sepharose beads and incubated overnight. Resin was washed and eluted proteins were resolved on a 7.5% Tris-HCl gel, dried overnight and visualized using a phosphorimager.

Analysis of expression of CYLD by quantitative PCR

Total cellular RNA was isolated using TRIzol (Invitrogen) reagent according to manufacturer's protocol. The cDNAs were generated in a reverse transcriptase reaction performed in 20 µl reaction volume containing 2 µg of total RNA. Relative quantitative analysis of CYLD mRNA expression was performed on a ABI PRISM 7300 sequence detection system (Applied Biosystems) in a total volume of 25 µl containing SYBR Green PCR master mix (2x), cDNA, and the following previously described primer sets: CYLD forward: 5'-TGC CTT CCA ACT CTC GTC TTG-3' and CYLD reverse: 5'-AAT CCG CTC TTC CCA GTA GG-3'¹²². Real time PCR results were normalized to GAPDH mRNA levels.

Co-expression of HPV E6 and CYLD

293T cells were transfected at 60-70% confluency in 6 well plates using FuGENE 6 Transfection Reagent with 1.2 µg of pcDNA3-CYLD-flag. Cells were co-transfected with either increasing concentrations of pKH3-16E6-HA (1µg, 2, µg and 4µg) or with one concentration of pKH3-11E6-HA (4µg). Cells were incubated for 48 hours, harvested in 1x lysis buffer and the proteins were resolved using gradient SDS-PAGE gels before being transferred to nitro-cellulose membrane. CYLD protein was detected using an anti-flag M2 peroxidase conjugate and immunoblots were developed using chemiluminescent reagents.

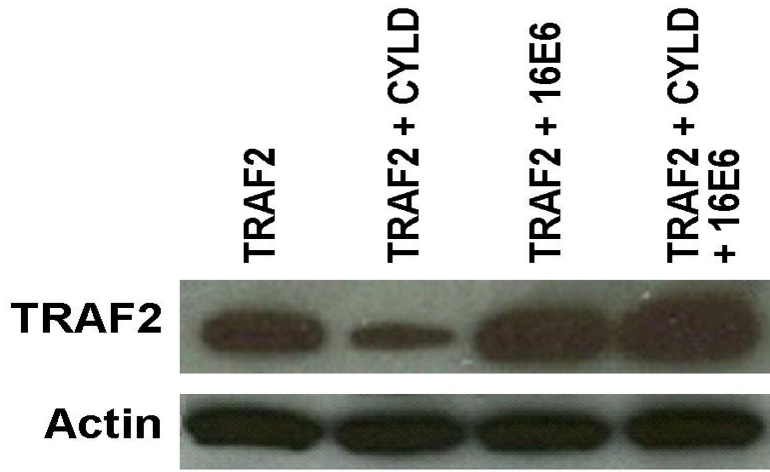
Results

Expression of CYLD in 293T cells reduces TRAF2 protein levels. CYLD-mediated loss of TRAF2 is inhibited by HPV16 E6.

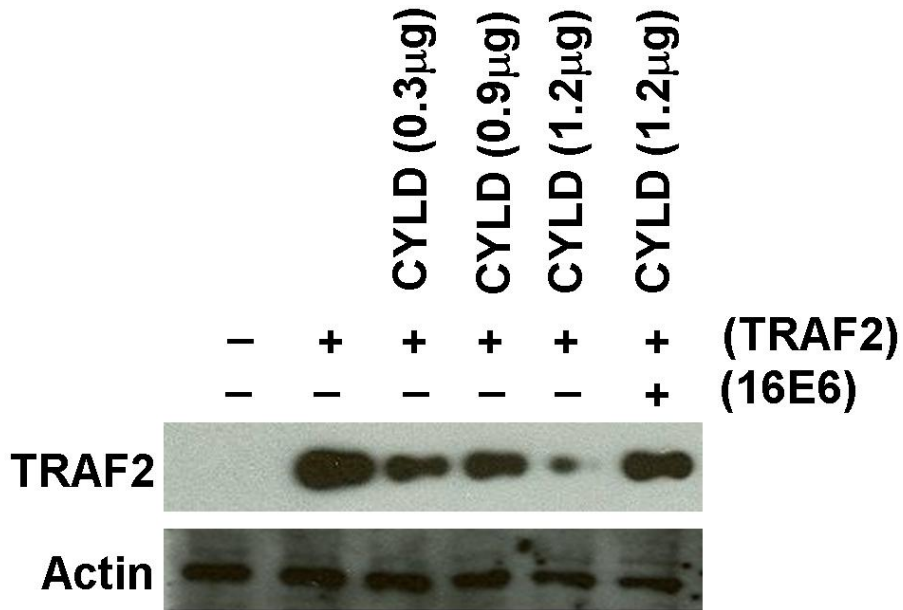
TRAF2 protein functions as a signal mediator upon activation of a variety of cell surface cytokine receptors and this leads to activation of an essential component of the NF- κ B pathway, the I κ B kinase (IKK). When plasmids expressing TRAF2 and CYLD were co-transfected into 293T cells, the amount of TRAF2 measured by western blot decreased dramatically (Fig. IV-1a, lanes 1 and 2). This CYLD-mediated loss of TRAF2 was increased with increasing doses of CYLD expression vector (Fig. IV-1b). Addition of an HPV16 E6 expression plasmid, but not an HPV11 E6 plasmid, blocked the loss of TRAF2 seen with CYLD expression (Fig. IV-1a, lane 4 and Fig. IV-1c, compare lanes 5 and 6). Thus, HPV16 E6 prevents CYLD-induced loss of TRAF2 in 293T cells

Figure IV-1. CYLD alters TRAF2 protein levels. (A) CYLD over-expression in 293T cells leads to loss of TRAF2. 293T cells were co-transfected with plasmids expressing CYLD-HA and TRAF2-flag. TRAF2-flag protein was detected using an anti-FLAG M2 peroxidase conjugate (Sigma); native TRAF2 was detected using an anti-TRAF2 peptide antibody (H249, Santa Cruz Biotechnology). (B) Loss of TRAF2 is dependent on CYLD dose. Plasmids expressing TRAF2-flag and increasing quantities of CYLD-HA expression plasmid were co-transfected into 293T cells. FLAG tagged TRAF2 was detected as describe above. (C) **HPV11 E6 does not inhibit CYLD-mediated TRAF2 loss.** Plasmids expressing TRAF2-flag and CYLD-HA were co-transfected into 293T cells with increasing quantities of HPV11 E6 expression plasmid. Western blot was completed using an anti-FLAG antibody.

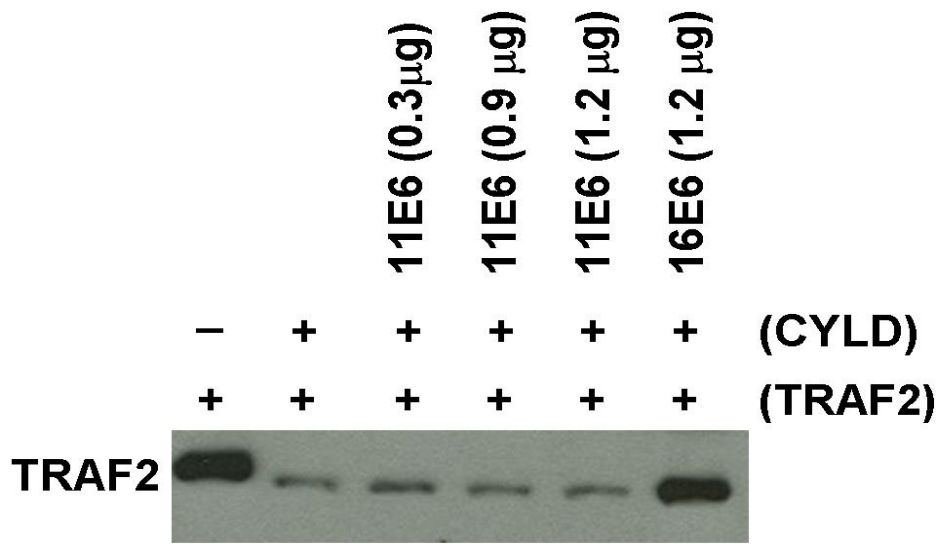
A



B



C

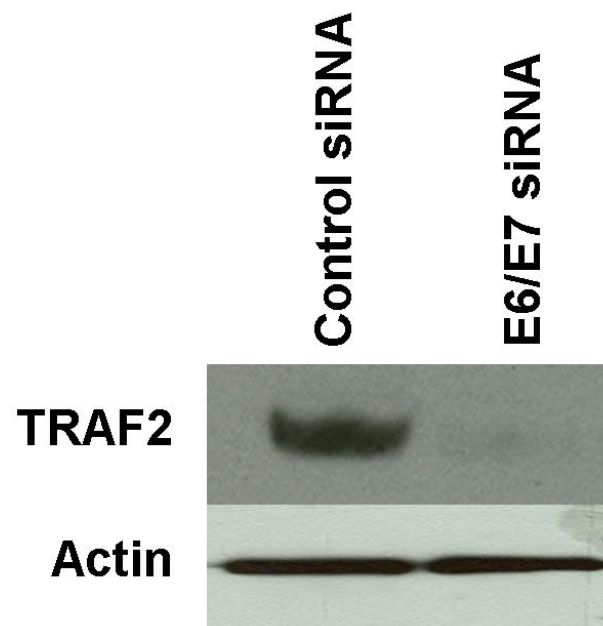


Silencing of endogenous E6 expression in SiHa cells leads to decreased levels of endogenous TRAF2.

The previous work with TRAF2 was completed using over-expression techniques. Here, we show that TRAF2 loss results from silencing endogenous HPV E6; supporting the hypothesis that CYLD-mediated modulation of TRAF2 occurs at physiological HPV E6 and CYLD levels and is not an artifact of protein over-expression. To determine whether endogenous levels of HPV E6 protein were sufficient to affect TRAF2 expression, TRAF2 levels in HPV 16-transformed SiHa cells were measured as a function of HPV E6 expression. For these studies, SiHa cells were transfected with an anti-E6/E7 siRNA or control siRNA. Following E6/E7 silencing, cellular TRAF2 levels were measured by western blot (Fig. IV-2). E6/E7 silencing reduced TRAF2 levels, indicating that endogenous levels of HPV E6 are sufficient to alter TRAF2 levels in HPV-transformed cells.

Figure IV-2. Silencing of endogenous E6 expression leads to loss of TRAF2.

SiHa cells were treated with anti-HPV16 E6/E7 siRNA or with a sequence-scrambled control siRNA. 48 hours after siRNA transfection, cells were lysed and the extracts subjected to SDS-PAGE. Proteins were resolved on an SDS-PAGE gel, transferred to nylon membrane and detected using an anti-TRAF2 antibody.



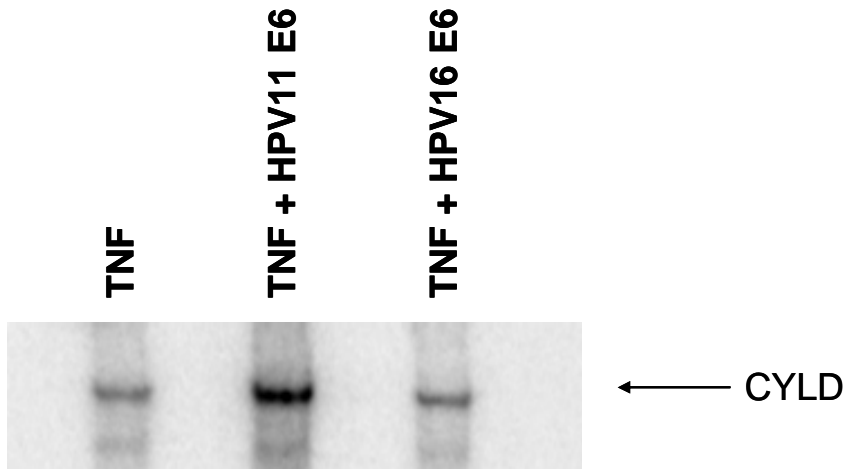
CYLD phosphorylation is increased by HPV E6 expression

The regulation of CYLD in the NF- κ B pathway is necessary for normal cellular response to stimuli. Phosphorylation is important for CYLD regulation and inhibition of CYLD-mediated deubiquitination of TRAF2 and NEMO (Fig. IV-3). We determined both HPV11 E6 and HPV16 E6 were able to increase CYLD phosphorylation. NEMO-mediated phosphorylation of CYLD may serve as a regulation mechanism to stimulate NF- κ B in cellular response after cytokine receptor stimulation.

Figure IV-3. HPV E6 proteins increase CYLD phosphorylation. (A)

Phosphorimage depicting phosphorylated CYLD. 293T cells were transfected with either HPV11 E6, HPV16 E6 or empty vector. Eighteen hours after transfection, [³²P] orthophosphate was added to the cell medium. Cell lysates were mixed with anti-CYLD antibody and protein A-sepharose beads. Resin was washed and eluted CYLD was visualized using a phosphorimager. **(B)** The radioactive counts detected by the phosphorimager for the image shown in A.

A



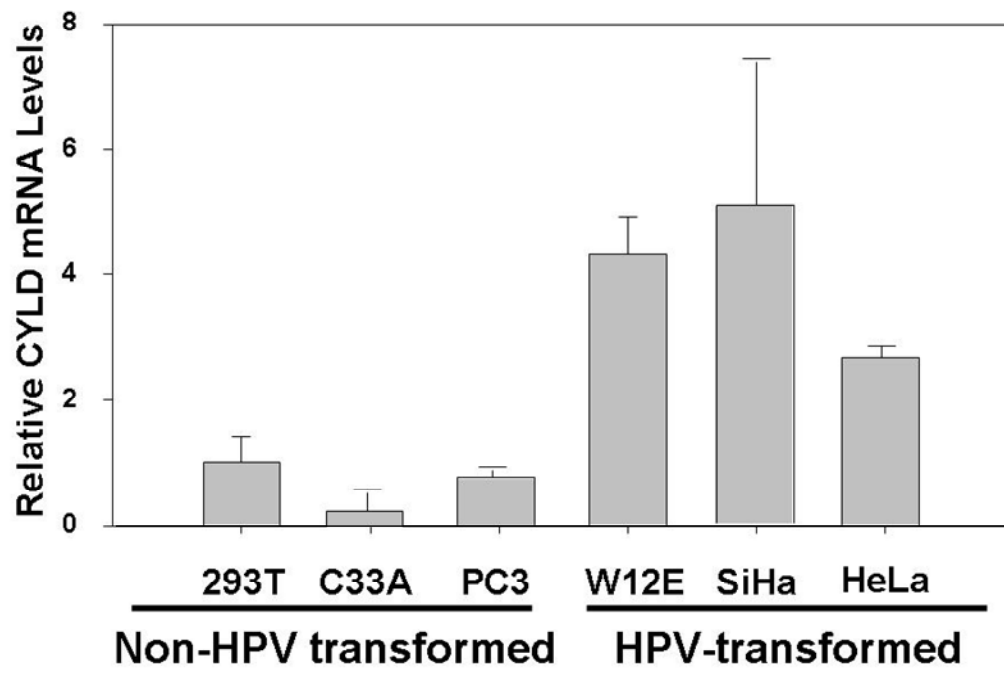
B

Sample	Counts detected by phosphorimager
Control plasmid +TNF	761260
HPV11 E6 + TNF	1443702
HPV16 E6 +TNF	1217169

CYLD mRNA levels in HPV-negative cell lines are lower than CYLD mRNA levels in HPV-transformed cell lines.

Given the fact that CYLD can function as a tumor suppressor, we investigated if some commonly used cultured cell lines had reduced levels of the CYLD mRNA. Using RT-PCR, CYLD mRNA levels in six cell lines were compared. Interestingly, the HPV-transformed cervical cancer cells lines (HPV positive cells lines), HeLa, SiHa, and W12-E expressed greater relative amounts of CYLD mRNA than the HPV-negative cell lines PC3, C33-A and 293T (Fig. IV-4).

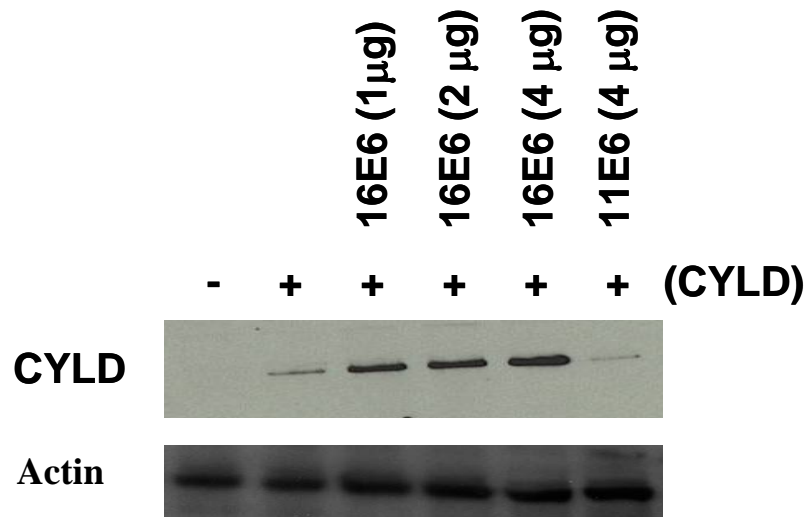
Figure IV-4. CYLD gene transcription levels vary between HPV-infected and non-HPV-infected cells. 293T, C33A, PC3, HeLa, SiHa, and W12E cells were harvested in 200 μ l of TRIzol buffer and processed according to manufacturer's protocol to isolate total RNA. CYLD cDNA was generated. Quantitative real-time PCR was performed; results were normalized to GAPDH mRNA levels.



HPV16 E6 protein prevents CYLD loss

After determining that HPV16 E6 and HPV11 E6 bind to CYLD, we hypothesized that this association may affect CYLD protein levels. To this end, we co-expressed CYLD with increasing concentrations of HPV16 E6. We observed that in the presence of HPV16 E6, more CYLD was detected than in the absence of HPV16 E6 (Fig. IV-5). We also determined that HPV11 E6 was unable to prevent CYLD loss. In cells co-expressing HPV11 E6 and CYLD, we did not observe the ability of HPV11 E6 to block CYLD loss, even at the highest concentration used of HPV16 E6 (4 μ g) (Fig. IV-5, compare lane 5 and 6).

Figure IV-5. HPV16 E6 protein expression increases CYLD. HPV11 E6-HA and CYLD-flag or HPV16 E6-HA and CYLD-flag were expressed in 293T cells. Cells were harvested and lysed. Proteins were separated using western blotting techniques and anti-FLAG antibody was used to detect CYLD.



Discussion

In an effort to identify how HPV E6 expression leads to NF- κ B pathway activation, we have found that the HPV16 E6 protein interacts with CYLD and inhibits CYLD's ability to promote the loss of TRAF2, a mediator of NF- κ B activation.

While it is well established that CYLD deubiquitinates TRAF2, we report the new observation that CYLD expression leads to loss of TRAF2 protein. Furthermore, when HPV16 E6 was co-expressed with CYLD, CYLD-mediated TRAF2 loss was inhibited (Fig. IV-1a). The expression of HPV16 E6 alone in 293T cells slightly increased TRAF2 levels. Thus, we propose that HPV E6 blocks CYLD-mediated TRAF2 loss. We also found that the loss of TRAF2 in 293T cells increased as a function of CYLD expression vector dose. As the quantity of CYLD expression vector used to transfect 293T cells was increased, the amount of TRAF2 loss also increased.

Expression of the low-risk HPV11 E6 protein did not inhibit CYLD-mediated TRAF2 loss. HPV16 and HPV11 E6 were both expressed independently and similar expression levels were observed (data not shown). Under conditions where HPV16 E6 was a potent inhibitor of CYLD-mediated TRAF2 loss (Fig. IV-1b), even high levels of HPV11 E6 failed to prevent CYLD-mediated TRAF2 loss. These observations are consistent with the differing pathogenic abilities and potentially differences in phenotypic clinical outcomes of the HPV16 and HPV11 E6 proteins.

Based on our data, and on work by others, we predict that upon stimulation of receptors by proinflammatory cytokines, TRAF2 is activated through lysine 63-linked auto-ubiquitination. Activated TRAF2 starts the classical signaling cascade resulting in NF- κ B translocation into the nucleus. We further predict that in the absence of HPV E6, CYLD binds and deubiquitinates K63-ubiquitinated TRAF2. Following K63 deubiquitination, TRAF2 becomes a substrate for the K48 ubiquitin ligase TRIP⁹⁶. TRIP-mediated K48 ubiquitination of TRAF2 leads to degradation of TRAF2 in the proteasome. This loss of TRAF2 would inhibit the canonical pathway for NF- κ B activation, leading to a reduction in nuclear NF- κ B. In the presence of HPV16 E6, CYLD-mediated deubiquitination of TRAF2 would be inhibited. K63-ubiquitinated TRAF2 would maintain the capability to interact with NEMO, thereby driving persistent canonical pathway activation and NF- κ B nuclear expression. The continued presence of the K63 ubiquitin chain could preclude K48 ubiquitination by TRIP, by altering the TRIP/TRAF binding observed by Lee *et al.*¹²³. Without TRIP-mediated K48 ubiquitination, TRAF2 would not be degraded in the proteasome, leading to TRAF2 accumulation and persistent NF- κ B activation. Such persistent NF- κ B activation would contribute to apoptosis resistance, would support HPV DNA replication, and would contribute to carcinogenesis of HPV-infected epithelial cells.

Because many of our experiments used over-expression of CYLD and HPV E6 proteins, it was important to determine if the NF- κ B-activating effects of HPV16 E6 occurred at physiological levels of HPV E6 and CYLD. To this end, we used SiHa cells to determine whether endogenous levels of CYLD and HPV16 E6

modulated cellular TRAF2 levels. SiHa cells are transformed with HPV16, and express very low levels of HPV16 E6. In keeping with our model that endogenous HPV E6 expression would inhibit CYLD-mediated TRAF2 loss, we found that treatment of SiHa cells with an anti-E6/E7 siRNA led to a marked decrease in total cellular TRAF2 (Fig IV-2). These results indicate that even the very small amounts of HPV E6 present in HPV-infected and HPV-transformed cells may be sufficient to increase TRAF2 levels significantly, leading to persistent NF- κ B activation.

CYLD phosphorylation was increased in the presence of HPV E6 proteins. This increase in CYLD phosphorylation was not great. However, work by others suggests phosphorylation of CYLD is essential for regulation of CYLD-mediated affect upon TRAF2 and NEMO. We hypothesize that E6-stimulated CYLD phosphorylation helps in suppression of CYLD-mediated TRAF2 and NEMO deubiquitination. Further study is needed to determine the significance and magnitude of the role of E6-stimulated CYLD phosphorylation in HPV E6-mediated suppression of CYLD inhibition of NF- κ B.

A growing body of evidence implicates CYLD as an important factor in tumorigenesis by inhibiting epithelial cell apoptosis^{87,88,95}. Consistent with this anti-apoptotic effect of NF- κ B, Hellerbrand *et al.* demonstrated reduced CYLD mRNA levels in human colon and hepatocellular carcinomas when compared to CYLD mRNA levels in normal tissue samples¹²⁴. Because HPV E6 protein-mediated inhibition of CYLD would have the same phenotypic effects as CYLD loss, we

hypothesized that HPV-negative cell lines, which lack HPV E6 to suppress CYLD activity, might have lower CYLD levels than HPV-transformed cells (in which the activity of CYLD is suppressed by HPV E6). Consistent with this hypothesis, we found low levels of CYLD mRNA in 293T, C33-A and PC3 cells when compared with the higher levels of CYLD mRNA in SiHa, HeLa and W12-E cells (Fig. IV-4).

Summary

CYLD is an ubiquitin protease that removes stimulatory K63-linked ubiquitin from TRAF2, an early activator of the canonical NF- κ B activation pathway. By deubiquitinating TRAF2, CYLD suppresses NF- κ B activation. We have found that CYLD expression in 293T cells leads to dose-dependent reductions in TRAF2 levels. This CYLD-mediated loss of TRAF2 is inhibited by co-expression of high-, but not low-risk E6 proteins.

We propose a model for the role of E6 in canonical NF- κ B pathway activation. By binding to CYLD, E6 blocks CYLD-mediated TRAF2 loss promoting NF- κ B activation. As a result of the HPV E6 and CYLD interaction, CYLD phosphorylation increases; a second mechanism that supports HPV-induced NF- κ B activation. By promoting NF- κ B activation by the canonical pathway, E6 proteins may drive

keratinocyte proliferation and prevent apoptosis, leading to the development of epithelial neoplasms.

V. Genetic variability in the HPV E6 open reading frame of HPV16 and HIV infected adolescents

Introduction

The HPV subtypes leading to carcinoma development are termed high-risk. Among high-risk types, type 16 stands out as the dominant contributor to carcinogenesis, being the type that causes over half of all cervical cancers¹²⁵. Why high-risk HPV are oncogenic while low-risk HPV types are not is unclear. Differences among the roles of the oncogenic proteins, E6 and E7, have long been hypothesized as the factors underlying the varying oncogenic potentials of high- and low-risk viruses¹²⁶.

The HPV E6 protein is a key player in HPV-induced cellular transformation, and is widely accepted as being the driving mechanisms of persistent HPV infections and dysplasia leading to carcinogenesis¹²⁷. The HPV E6 protein is a small polypeptide of approximately 150 amino acids, containing two zinc-finger motifs that are essential for proper protein folding^{128,129}.

Given the complexity and diversity of HPV E6 cellular interactions with host cell proteins (see chapter 1 for review), we postulate that any differences in HPV16 E6 DNA sequence may alter HPV16 E6 interactions with other proteins. Numerous studies suggest there are distinct HPV E6 variants within HPV types, and that specific genetic variations among HPV type 16 E6 proteins may correlate with, and

potentially serve as genetic markers of, risk for the development of cervical intraepithelial neoplasia and cervical cancer^{130,131}.

HPV infections are common in sexually active women, with estimates of over 80% of women will be infected in their lifetimes^{132,133}. Active immune systems are effective in protecting the majority of these women from life-threatening HPV disease. Nonetheless, immunocompromised individuals, particularly those with human immunodeficiency virus (HIV) driven immuno-suppression, are at increased risk for acquiring HPV, and frequently develop persistent HPV infections¹³⁴. Additionally, the sexual practices that increase risk for acquiring an HIV infection also increase risk for acquiring HPV infections. As a result of immunodeficiency and sexual behavior risk factors, HIV-infected women have increased prevalence of cervical HPV^{135,136}, anal HPV^{137,138}, and oral HPV¹³⁹ infections. There is an inverse relationship observed between anogenital HPV infections and CD4+ levels¹³⁴. Studies to date have not identified HIV as having a direct causative role in HPV pathogenesis, however, HIV-induced immune system suppression contributes to a cellular environment that allows for HPV persistence and tumorigenesis¹³⁴.

In HIV infected women, HPV infections persist because the decreased clearance by the immune system. Thus, in women with decreased immune responses, there could be acquisition and persistence of HPV at higher rates resulting in the women harboring HPV E6 with greater genetic diversity. The identification of novel HPV E6 sequences could start to explain why HPV in HIV infected women experience increased HPV transformation and HPV mediated neoplasia.

We hypothesized that immune compromised women infected with HIV would harbor novel HPV E6 sequence variations when compared to women not infected with HIV. The immune compromised individual may serve as a host with reduced selective pressure on HPV E6 allowing mutations to appear in non-essential parts of the HPV E6 protein, parts that do not interact with p53 and CYLD. To test this hypothesis, we performed HPV16 E6 PCR on cervical lavage specimens from forty-six adolescent female patients of the REACH Cohort at the University of Alabama who were infected with HPV16 and HIV. We identified HPV E6 sequences that contained amino acid differences when compared to the HPV E6 reference sequence in the samples from this cohort of women. All of the HPV E6 sequence amino acid variations detected had been previously identified and published in HIV negative women infected with HPV16. These findings did not support the hypothesis that adolescent women co-infected with HPV16 and HIV harbor more HPV E6 sequence variants than HIV negative women.

Methods

Acquisition and preparation of clinical specimens

Clinical specimens were obtained from adolescent women undergoing gynecological procedures as participants in the REACH program administered by the University of Alabama at Birmingham. Samples were obtained by vaginal lavage. The wash specimens after collection were frozen and stored in -80°C until DNA extractions and PCR analysis were completed. A total of 46 cervical specimens of

HPV 16 positive adolescent females were obtained. Presence of the HPV 16 was confirmed by northern blot hybridization. All participants from whom samples were collected were women co-infected with HIV.

Detection of HPV E6 DNA using PCR

Polymerase chain reaction (PCR) was used to detect and amplify HPV16 E6 DNA in the cervical wash specimens. All wash samples were used as PCR template using a pair of primers designed to anneal to HPV16 E6 prototype (HPV 16R) (GenBank K02718): forward (5' ata tac tat att ttg tag cgc cag gcc cat 3') and reverse (5' ccc ctt ata tta tgg aat ctt tgc ttt ttg 3') (Figure V-1). The primer pair was first verified and annealing temperature optimized by using the primer pair in a temperature gradient PCR amplification of the HPV 16R prototype containing plasmid.

Once verified, the primer pair was used to detect and amplify E6 DNA from the cervical wash specimens using a PCR reaction mixture consisting of 10 µl of specimen DNA, 10x PCR buffer, 1.5mM MgCl₂, 200 µM total of each deoxynucleoside (dNTPs), 10 µM of specified forward and reverse primers and 1 U of Platinum Taq DNA polymerase (Invitrogen) in a final volume of 50 µl. HPV E6 DNA was amplified by PCR protocol as follows: an initial denaturation for 3 minutes at 95°C, then for 35 cycles, denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, elongation at 68°C for 1.5 minutes and a final elongation at

68°C for 3.5 minutes. If the initial PCR was unsuccessful, the amplification was attempted using the less stringent primer set two (Fig. V-1).

The amplified HPV16 E6 product (a 987 base pair product was expected) was applied to an agarose gel infused with ethidium bromide, and single band samples were sent for sequencing. Of the samples without clear single bands, a sample was collected from the band of expected size using a pipette tip inserted into the agarose gel. The DNA in the excised gel sample was purified using Qiaex II agarose gel extraction kit (Qiagen) according to manufacture protocol. The purified DNA was used in a second round of PCR with primer set three (Fig. V-1) according to previously stated conditions, and again subjected to electrophoresis in an ethidium bromide agarose gel before being sent for sequence analysis.

Figure V-1. Primer sequences used for PCR amplification of the HPV 16 E6 gene. Primer set one was designed to anneal to E6 open reading frame of HPV 16 prototype (HPV 16R). Primer set two is a truncated version of primer set one. Primer set three was designed to anneal to E6 open reading frame interior to primer set one. Primer set one was is expected to result in a 987 base pair product.

Primer set 1

Forward

5' ata tac tat att ttg tag cgc cag gcc cat 3'

Reverse

5' ccc ctt ata tta tgg aat ctt tgc ttt ttg 3'

Primer set 2

Forward-truncated

5' at att ttg tag cgc cag gc 3'

Reverse-truncated

5' ccc ctt ata tta tgg aat c 3'

Primer set 3

Forward #2-downstream

5' cgg ttg cat gct ttt tgg 3'

Reverse #2-upstream

5' cac aac ggt ttg ttg tat tgc 3'

Sequence analysis of HPV-16 E6 open reading frame

Sequencing was completed by North Carolina State University (Raleigh, North Carolina) and Duke University (Durham, North Carolina) sequencing centers using standard procedure and primer set one described above. The sequences were compared to HPV16R reference sequence using alignment software, BLAST. Based upon alignment with HPV16R published sequence, variants were identified as having at least one amino acid difference in the HPV E6 sequence.

Results

A total of 46 cervical wash specimens were analyzed from samples collected of adolescent females. Of the 46 specimens, HPV E6 DNA sequence was detected and amplified from 18 samples (39.1%). Of the 18 samples from which we could amplify a DNA product, 12 samples corresponded to HPV 16R. Of the HPV16 E6 sequences that contained mutations, all corresponded to mutations known of previously characterized HPV clades.

Sequence variations of the HPV 16 E6 oncogene

In the samples with mutations of the prototype HPV 16E6, a total of twelve nucleotide point mutations were noted. The HPV E6 nucleotide polymorphisms observed and their prevalence in this sample population were: a change from T to C at nucleotide 84 (T84C) (n=1), A131G (n=1), G132T (n=1), C143G (n=1), G145T

(n=2), C335T (n=2) and T351G (n=4); n total=12 (table V-1). There was one common nucleotide alteration at position 351, a change from T to G was observed in 4 of the samples tested.

The eleven nucleotide sequence changes observed resulted in 10 amino acid changes. The HPV E6 amino acid variations observed and their prevalence in this sample population were: a change from R to G at amino acid 10 (R-10-G) (n=1), R-10-I (n=1), Q-14-D (n=1), Q-14-H (n=2), H-78-Y (n=2), L-83-V (n=4); n total=11 (table V-2). The L-83-V variants appeared the most frequently, being present in 4 of the 7 samples. Three of the samples (samples 3, 11, and 31) harbored mixed variant infections (Table V-2). Sample 11 actually contained four variants, and only lacked the most abundant variant L-83-V detected in all the other four samples.

Table V-1. Nucleotide comparison of the different HPV 16 E6 gene polymorphisms. The HPV E6 nucleotide of the reference is shown. The samples with nucleotide changes are listed along with the substitute nucleotide.

HPV 16 E6 open reading frame nucleotide position and polymorphisms

Nucleotide position	84	131	132	143	145	335	351
---------------------	----	-----	-----	-----	-----	-----	-----

Reference sequence nucleotide	T	A	G	C	G	C	T
-------------------------------	---	---	---	---	---	---	---

Assigned specimen number							
3					T	T	G
11			T	G	T	T	
16							G
19							G
31		G					G
32	C						

Table V-2. Observed HPV E6 amino acid variations. Samples containing the listed variant are indicated by 'X'.

	Variant R10G	Variant R10I	Variant Q14D	Variant Q14H	Variant H78Y	Variant L83V
Sample 3				X	X	X
Sample 11		X	X	X	X	
Sample 16						X
Sample 19						X
Sample 31	X					X

Discussion

Many studies have shown that individuals infected with HIV often have increased rates of detectable HPV DNA. Sun *et al.*¹⁴⁰ observed that HIV infected females had higher rates of HPV-16 infections compared to control group of non-HIV-infected women. Given immune system suppression in HIV-infected women, there could exist previously uncharacterized HPV E6 variant maintained with the immunocompromised host. All of the different variants detected in our study (Table V-2) have also been recognized in non-HIV infected populations and thus, no novel variants were observed. A consistent confounding issue in our study and other studies attempting to characterize HPV E6 variants is sample size. Chaturvedi *et al.* studied analyzed 24 samples¹⁴¹ and Perez-Gallego *et al.* studied 48 samples¹⁴² from HIV infected individuals. Neither of these two studies revealed previously uncharacterized variants.

In this study, we identified that HIV-infected adolescent females co-infected with HPV16 harbor HPV16 E6 nucleotide sequence polymorphisms. All the nucleotide polymorphisms detected have been previously reported. A polymorphism resulting in an amino acid switch constitutes a HPV16 variant. HPV16 lineage groups or clades have been described to categorize the different HPV16 variants; namely African, North American, Asian American, and European. The majority (four out of six different variants) of the HPV16 variants we observed belonged to the African clade. The African clade variants were R10I, Q14D, Q14H, and H78Y. One variant R10G belongs to the European clade and one variant, L83V, belongs to

the North American clade. The North American clade variant L83V had the highest appearance frequency among the different samples; as all except one sample contained this variant. No variants belonging to the Asian clade were observed in our samples. We hypothesize the detection of variants belong to the African clade and North American clade in our samples is a result of the population in which the samples were obtained, women living in Birmingham, Alabama. Ethnicity data was not provided with the samples, however, it is estimated that this area has a significant African American population.

The inability to detect novel HPV variants in our samples suggests two things. First, the selective pressure against mutations of HPV E6 remains high, even in immunocompromised individuals. HPV can evade detection and clearance by the host immune system due to the natural history of HPV; thus the host immune system may not contribute to the pressure against mutations of HPV E6. Alternately, we could conclude that the rate of HPV E6 mutation is extremely low; so low that it would not be apparent in a single organism with decreased immunity. HPV is a DNA virus and the rate of mutations is reduced when compared to RNA viruses. If the rate of mutations in HPV is low, then the rate of HPV E6 mutations would also be low.

This study using HPV and HIV co-infected women showed high correlation with a number of other studies that have also shown that HPV E6 intratypic amino acid variants can be detected among immuno-competent (HIV negative) HPV infected women. The majority of the E6 amino acid switches detected in our samples were located at amino acid residues 10, 14 and 83; of the eleven total amino acids

switches noted, 9 occurred at either residue 10, 14 or 83. These ‘frequently mutated’ residues coincide with findings obtained from work completed by Da Costa *et al.* who sequenced 628 HPV16 positive anal specimens¹⁴³. Of the five different HPV E6 samples harboring variations (samples number 3, 11, 16, 19, 31; table V-I) the L-83-V variant was present in four of the samples, making it the most prevalent. Qui *et al.* also observed the L-83-V variant in 35 of 114 samples from cervical cancer patients in Sichuan China, the most abundant of the detected variants¹⁴⁴.

HPV E6 variants have been identified by a number of researchers as appearing in high proportions in progressively more invasive dysplasia. The L-83-V variant was detected in 4 of our samples. This variant L-83-V was detected either alone or with other HPV E6 sequence variants in 44% of CIN III, and 88% of invasive cervical cancer according to work by Zehbe¹⁴⁵. Zehbe *et al.* also concluded that the L-83-V presence may contribute to the progression of CIN I to CIN III and invasive cervical cancer¹⁴⁵. Similarly, the L-83-V variant was associated with HPV infection persistence over HPV clearance when compared to the reference amino acid leucine at the 83 position in samples obtained from a cohort of French women in a study by Grodzki *et al.*¹⁴⁶. The L-83-V HPV E6 promoted HPV-induced transformation over reference¹⁴⁷ in calcium triggered keratinocyte differentiation studies¹⁴⁸.

Functional studies in which various regions of the HPV E6 protein were mutated demonstrated alterations in protein regions containing residues 10 and 78. Mutations in regions containing residues 10 and 78 have been shown to disrupt

known transforming biochemical functions of the HPV E6 protein (reviewed in¹⁴⁵). In this present study we identified mutations in residues 10 and 78.

Changes in HPV E6 regions with biochemical functions may contribute to the HPV variant being better able to lead to malignancy. Lee *et al.* even claimed that of the HPV proteins, sequence polymorphisms contained within HPV E6 DNA are the most beneficial in predicting progression of dysplasia¹⁴⁸.

By identifying HPV variants associated with aggressive disease, we can then start to examine what biological processes are altered in that variant. With the current strides in genome wide screening, the results of large epidemiological studies identifying potential variants associated with increased disease may be incorporated into the genome technology for development of new diagnostic and therapeutic strategies.

A problem with this study was that most of the specimens failed to yield a PCR product. Of the 46 samples, we were only able to amplify HPV16 E6 DNA from 18 samples. The initial sample size of 46 was not large, and the number of samples in which we were able to amplify the DNA for sequencing was small.

Additionally, some studies attempting to link HPV E6 variants with disease progression have shown results in contrast to other published works. The obvious thoughts to explain these differences included issues with sample size and differences in populations based upon geographical regions

Summary

Co-infection with HIV is associated with increased rates of HPV mediated transformation and malignancy development. We predicted that the HPV E6 in HIV and HPV co-infected women would harbor novel HPV E6 mutations. To this end, we analyzed HPV E6 protein DNA sequences from HPV type 16 and HIV infected females. We only detected the presence of previously recognized E6 variants, suggesting that HPV E6 sequence mutations are conserved even among the immunosuppressed.

VI. Discussion and Future Directions

HPV infections are common, however, the majority of us never know of our HPV status, as the majority of HPV infections show no symptoms. Of the cases that present as anogenital warts, the greatest issues to combat are often the psychological/social anxiety and physical nuisances presented just by the presence of the wart. However, in a small subset of the female population in the US and in a significantly greater number of women worldwide, HPV infections can be life-threatening. Even though HPV has been characterized for some time, our understanding of the virus's lifecycle and oncogenic mechanisms needs further elucidation.

The observations around HPV actions upon the p53 tumor suppressor protein were groundbreaking, and provided molecular support to observations between HPV infection and malignancies. However, immortalization and oncogenesis-related functions are complex. How HPV leads to malignancies cannot be completely explained with only the classical carcinogenetic factors such as the HPV E6 and p53 association and the HPV E7 and pRb relationship. Liu *et al.* performed a series of experiments investigating three known protein partners of HPV E6; E6-AP, p53 and E6BP. These authors showed that these associations were not sufficient to explain how HPV E6 to drives immortalization of mouse mammary epithelial cells¹⁴⁹. In

studying HPV pathogenesis, we sought to further our understanding of HPV E6 protein involvement in HPV oncogenesis.

As with most carcinoma development, one mutation or deleterious event is not sufficient for carcinogenesis. Several events factor into the development of HPV driven malignancies. Some of the risk factors are age of onset of sexual activity, the number of sexual encounters, cigarette smoking, and immune suppression. A person's immune status and genetic background are also factors. How these non-viral risk factors contribute to the development of HPV-induced malignancies are areas of great debate and research. Nevertheless, the ability of HPV to integrate into the host genome is an essential characteristic associated with transformation potential. HPV integration promotes persistent HPV E6 expression and HPV E6 of high-risk HPV types contribute to HPV-induced malignancies.

HPV E6 integration resulting in constitutive expression is important in HPV driven malignancies because the HPV E6 proteins target several proteins that promote apoptosis, such as E6AP, Bak^{150,151}, FADD¹⁵², c-Myc^{153,154}, and procaspase 8¹⁵². HPV E6 protein also leads to the degradation of PDZ-containing proteins through HPV E6 protein C terminus^{155,156}. PDZ-containing proteins are known to contribute to cell signaling and cellular adhesion properties. HPV E6 also supports chromosome maintenance through interaction with telomerase reverse transcriptase hTERT. Similarly, HPV E6 binds E6-BP, thereby contributing to cellular proliferation¹⁵⁷.

All these proteins associating with HPV E6 and binding partners of HPV E6 contribute to the prevention of cell senescence and promotion of cell proliferation in cells expressing HPV E6. Nonetheless, cells can respond to unscheduled proliferation by inducing apoptosis. NF- κ B expression prevents apoptosis as NF- κ B up-regulation is often seen in malignant cells. NF- κ B activation results in transcription of a variety of genes involved in proliferation, immune and inflammation responses, and protection against apoptosis; all factors that contribute to tumor formation in keratinocytes^{97,98}. HPV infection leads to activation of NF- κ B and thereby circumvents cell apoptotic signals.

In keratinocytes, a regulator of classical NF- κ B pathway activation is the CYLD protein. CYLD was initially identified when mutations in the protein resulted in a predisposition to multiple, frequently benign, tumors of skin appendages^{158,159}. CYLD deubiquitinase function inactivates TRAF2 and NEMO. NEMO and TRAF2 are essential players in the signaling complexes leading to NF- κ B activation (see Chapter II and Chapter IV for full review). Inhibition of these substrates by CYLD results in NF- κ B attenuation^{87,95,88} (Fig. II-1).

In the work presented in this dissertation, we demonstrated that the HPV16 E6 binds CYLD. The degradation of p53 by HPV E6 is mediated through the ubiquitin ligase E6AP, so it is not surprising that HPV E6 interacts with another ubiquitin-related protein. We propose the HPV16 E6 and CYLD association activates the canonical NF- κ B pathway in HPV-infected and HPV-transformed cells by interfering with CYLD, potentially through HPV enhanced phosphorylation of

CYLD, and preventing CYLD from suppressing NF- κ B activation. Alternatively, HPV E6 could block CYLD/TRAF2 association, or HPV E6 could block CYLD/NEMO association. Ultimately, this is important because the HPV16 E6/CYLD interaction provides a molecular explanation to the observed HPV induced activation of NF- κ B. HPV E6 driven inhibition of CYLD-mediated suppression of NF- κ B activation appears to be a p53 independent function of HPV E6.

A recurring observation is that low risk HPV E6 proteins exhibit decreased binding affinities to a number of binding partners of high-risk HPV E6, such as the proteins listed above¹⁶⁰. In this study the HPV E6 protein from the low-risk HPV type 11 was able to bind CYLD. Consistent with other studies comparing high-risk E6 to low-risk HPV E6, HPV11 E6 lacked the ability in our assays to mediate suppression of CYLD biochemical activities, including suppressing CYLD-mediated NF- κ B inhibition and TRAF2 loss (Fig. III-1b and Fig. IV-1c, respectively).

After we determined that HPV16 E6 would activate NF- κ B expression, we worked to identify the underlying molecular mechanism. We demonstrated that CYLD leads to loss of TRAF2 protein. It had previously been reported that CYLD deubiquitinated TRAF proteins⁸⁷ and our results indicate a possible consequence to TRAF2 deubiquitination. In our study, we demonstrated the ability of HPV16 E6 to protect TRAF2 from CYLD-mediated degradation. HPV16 E6's capacity to protect

TRAF2 from CYLD-mediated degradation, further solidifies a molecular basis for HPV-induced NF- κ B activation.

We have shown that HPV E6 proteins binding to CYLD leads to HPV-mediated activation of the NF- κ B pathway and identified TRAF2 loss as a consequence of the CYLD and HPV16 E6 interaction. Correlating our data with the findings of others, we are able to construct a model for how HPV E6 mediates NF- κ B canonical pathway activation (Fig VI-1). HPV16 E6 binds the CYLD protein. The HPV16 E6 and CYLD association prevents CYLD-mediated inactivation of TRAF2 and NEMO. This is possibly accomplished by preventing CYLD-mediated deubiquitination of TRAF2 and NEMO. Activation of TRAF2 and NEMO stimulates assembly and activation of the IKK complex. The active IKK complex results in NF- κ B translocation (compare Fig. VI-1 and Fig. II-1). Figure VI-1 illustrates our model of how attenuation of CYLD activity by E6:CYLD complex formation underlies HPV-mediated activation of NF- κ B in HPV-infected and HPV-transformed cells.

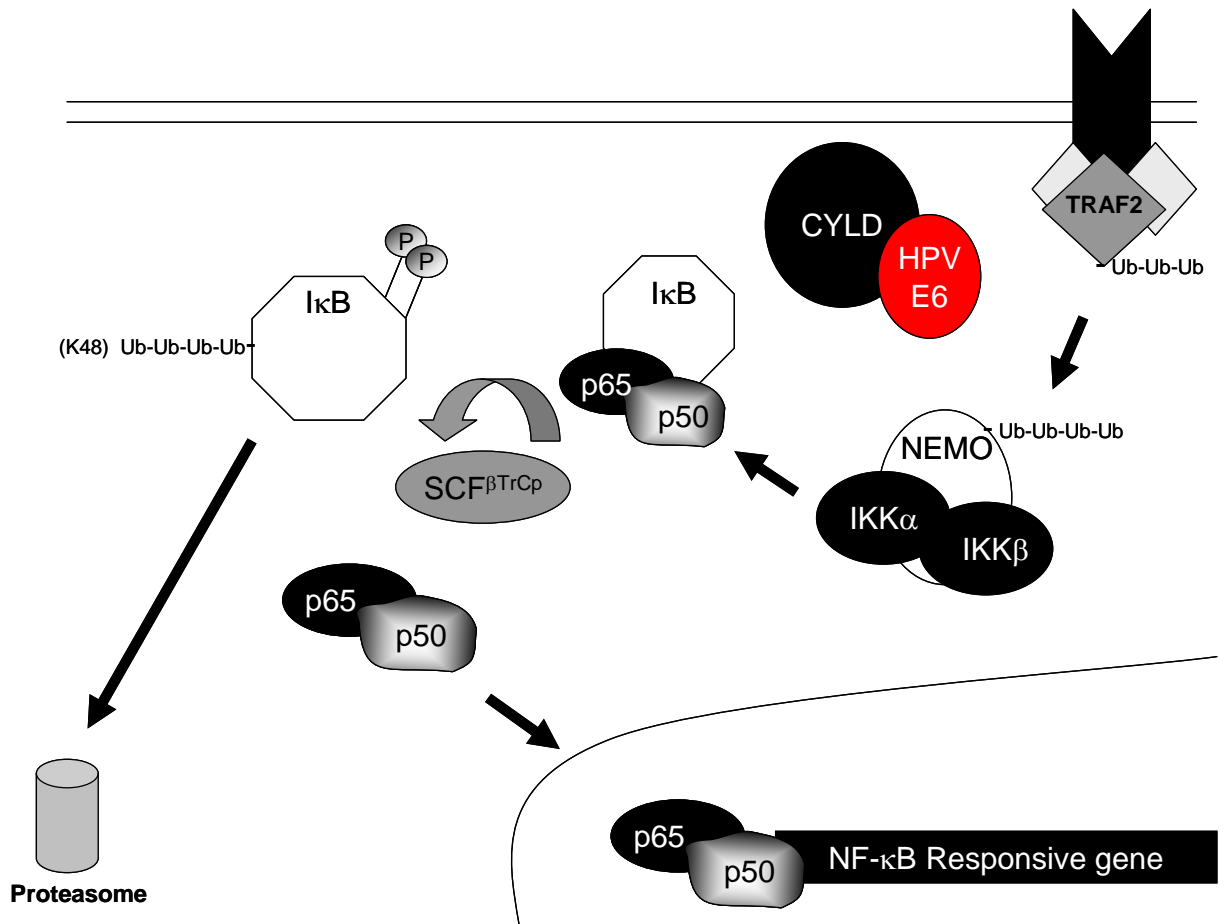
HPV transformed cells are dependent upon HPV E6 expression for continuous activation of NF- κ B resulting in apoptosis resistance. NF- κ B expression is essential to a number of cancers, including colon, hepatocellular, cervical and some head and neck cancers. In colon and hepatocellular carcinoma cell lines the CYLD protein is down regulated or lost stimulating constitutive NF- κ B activity that results in proliferation of these cell lines¹²⁴. CYLD silencing promotes increased NF- κ B activation and increased apoptosis resistance^{87,88}. We showed increased CYLD mRNA levels in HPV infected and transformed cell lines when compared to non-

transformed and transfected cell line (Fig. III-5). One hypothesis is that these cervical cancer cells are up-regulating CYLD suppressor protein to reduce continuous NF- κ B; however, the continuous expression of HPV16 E6 prevents CYLD-mediated inhibition of NF- κ B activation, thereby stimulating NF- κ B transcriptional activity, and resulting in the forging of apoptosis resistance.

Work by An *et al.*¹⁶¹ studying hypoxia-induced NF- κ B pathway activation identified that HPV transformed cell line exhibited NF- κ B activation; and subsequently, identified that silencing high risk HPV E6 resulted in inhibition of NF- κ B activation under hypoxia-induced conditions. Furthermore, these researchers demonstrated that under hypoxic conditions, HPV E6-mediated activation of NF- κ B was dependent upon CYLD. They proposed a model where HPV E6-mediated ubiquitination of CYLD inhibits CYLD suppression of NF- κ B activation. These listed experimental observations by An *et al.* support our observations that HPV16 E6 association with CYLD leads to abatement of CYLD-mediated NF- κ B activation. Our data showed that the HPV16-E6/CYLD association modulates CYLD's effects upon TRAF2 and suggest a role for HPV E6-mediated phosphorylation in CYLD regulation. An *et al.* also observed that HPV E6 expression reduced CYLD levels and suggested that HPV E6-mediated reduction of CYLD happened in proteasomal-dependent mechanism leading to degradation of CYLD. In our co-expression assay, we observed that HPV E6 expression did not reduce CYLD. Instead, we observed

that the presence of HPV16 E6 lead to an increase in CYLD protein levels (Fig. IV-5).

Figure VI-1. Schematic showing HPV16 E6 involvement in NF- κ B signaling pathway. This schematic displays a working model for HPV16 E6 involvement in NF- κ B activation. HPV16 E6 binds CYLD. CYLD associated with HPV16 E6 is unable to deubiquitinate activated TRAF2 and NEMO. Ubiquitinated TRAF2 activates IKK. Active IKK leads to phosphorylation-mediated proteasomal degradation of I κ B. NF- κ B (p56/p50), no longer bound by I κ B, is free to translocate into the nucleus. Overall, in the presence of HPV16 E6, CYLD is bound and unable to inhibit NF- κ B pathway activation.



Therapeutic potential of NF- κ B inhibitors

NF- κ B activation has a major role in our current understanding of the multiple triggers required for progression to cancer. NF- κ B functions as a transcription factor that up-regulates factors responsible for cellular proliferation. Thus, inhibition of NF- κ B could be used as a means to inhibit tumorigenesis. Given the fact that HPV needs actively replicating cell for viral replication, NF- κ B attenuation could be used to stop viral replication. Work in our lab has preliminarily shown that blocking NF- κ B leads to cell death in HPV-transformed cells (data not shown).

NF- κ B is also a regulator of inflammatory responses. Inflammation enhances pathogenesis of many cancers¹⁰⁵. Chronic inflammation has been recognized as a hallmark indicator of tumorigenesis^{100,162}, particularly in epithelial derived tumors¹⁶³, cervical intraepithelial neoplasia¹⁶⁴, and cervical cancer¹⁰¹. Chronic HPV infections are often observed in tandem with chronic inflammation. One reason for this correlation could be that HPV reduces CYLD-mediated suppression of NF- κ B activation, thereby up-regulating NF- κ B and the inflammatory genes controlled by NF- κ B transcription. We hypothesize that the persistent NF- κ B activation caused by E6-mediated inhibition of CYLD advances carcinogenesis in HPV-infected cells by enhancing epithelial inflammation. Improved understanding of the role of chronic inflammation in the development of HPV driven tumorigenesis is required before we can begin to fully appreciate the power of the HPV E6 and CYLD interaction.

A future product of this work could be the creation of therapeutics designed to reduce NF- κ B activation. A rational strategy would be to interfere with activation of IKK, since IKK- α and IKK- β are highly specific for phosphorylation of proteins required for NF- κ B activation, thus inhibitors of IKK could be effective against cancer and inflammatory diseases⁶³.

HPV and HIV infection in patients

Patients with decreased cytotoxic T-lymphocyte mediated responses are at a high risk of symptomatic and persistent HPV infection^{32,165}. Sun *et al.* reported that HIV positive women have higher rates of persistent HPV16 infections than non-HIV infected women¹⁶⁶. Regression of HPV-mediated transformation is greatest with higher functioning CD4+T cells. The degree of HPV persistence correlates with CD4 T cell levels¹⁶⁶.

We hypothesized that women co-infected with HPV and HIV would exhibit greater genetic variability in the HPV E6 open reading frame than HPV infected women without a co-infection with HIV. HIV infected women with immune suppression have reduced HPV viral clearance and may have HPV E6 variants not detectable in women with normal immunity. We found HPV16 polymorphisms within the samples from which we were able to detect and sequence HPV E6 open reading frames. We did not find HPV E6 variants that have not been previously characterized in non-HIV infected populations.

Some of the HPV E6 variants detected in our investigations, even though they are not novel, may be worth further study because other researchers have observed correlations between these HPV E6 variants and progression of HPV infections to CIN III and cancer. This is important because the HIV-mediated immunocompromised women already are at greater risk of CIN progression because of reduced clearance of HPV¹⁶⁷. The combination of reduced HPV clearance due to being HIV positive and infection with a more virulent HPV E6 variant could be sufficient for aggressive carcinoma development.

Future Directions

This dissertation provides a foundation for future studies of how HPV-infected and HPV-transformed cells activate the NF- κ B pathway.

Determining the molecular mechanism behind HPV-mediated activation of NF- κ B

Identification of binding domains for both HPV E6 and CYLD could start to elucidate the nature of the interaction and the biochemical consequences of the association. The zinc finger motifs on HPV E6 and the CAP-gly domains on CYLD are potential candidates as these areas are previously characterized as being essential for noted molecular function with other substrates. To confirm further that a particular domain of CYLD is sufficient to bind HPV E6, truncations of CYLD could be assayed for binding ability to HPV E6.

To better understand the HPV E6 and CYLD association, we could create truncations of CYLD and CYLD mutants with point mutations in CYLD's NEMO binding site, TRAF2 binding site and in the deubiquitinase activity domain of CYLD. These CYLD mutants would be used in our dual luciferase assay to measure NF- κ B activity in the absence and presence of HPV E6 (the dual luciferase assay was previously described in chapter III). This study would assist in further defining the HPV E6 and CYLD association in NF- κ B modulation.

CYLD expression in 293T cells resulted in the loss of TRAF2. Further work needs to be completed delineating the cellular mechanism underlying CYLD-mediated TRAF2 loss and rescue by HPV16 E6. CYLD binds TRAF2 normally and deubiquitinates TRAF2. We can determine if HPV E6 interferes with the binding of CYLD to TRAF2. Using a co-immunoprecipitation assay, we can determine if the binding of CYLD to TRAF2 is altered in presence the HPV E6. Measuring CYLD binding affinity for TRAF2 in the presence of HPV E6 will help to understand if the binding of HPV E6 contributes to a physical blocking of the TRAF2 binding site on CYLD.

It will also be important to study the role of HPV E6 on the non-classical NF- κ B signaling pathway. This would be done by defining the importance of the HPV E6 and Bcl-3 interaction. Bcl-3 is deubiquitinated by CYLD. Ubiquitinated Bcl-3 mediates processing of NF- κ B1 and NF- κ B2. Processing of NF- κ B1 and NF- κ B2 is required for Bcl-3 activation of NF- κ B heterodimers Bcl-3/p52 and Bcl-3/p50 (see Table II-1 and Chapter II for review).

HPV in the HIV positive women, what now?

We would like to further examine this notion that HIV infected women harbor greater genetic diversity of the HPV E6 sequence. Our sample size was low, so this study should be repeated with a larger cohort of women. The conclusion of this larger study could mirror our current finding of no observed increase in variants

among the HIV positive and negative HPV-infected women. We would then start to explore other factors that contribute to increased prevalence and persistence of HPV in HIV patients.

There exist a number of recognized HPV 16E6 intratypic variants (varying about 2% from designated reference sequences). These intratypic variants have different abilities to modulate HPV E6-mediated actions; resulting in varied HPV E6 oncogenic potential^{131,168} Furthermore, Stoppler *et al.* expressed variant HPV E6 proteins and p53 for binding studies, and showed that the variants with alterations in the amino terminus of HPV E6, when compared to the reference HPV E6, sequence had different binding affinities for p53¹⁶⁹. The amassing of variant data is essential to finding any genetic sequence differences that correlate with cervical intraepithelial neoplasia (CIN) and CIN progression to cervical carcinoma.

In an effort to correlate HPV-16 E6 sequence variations with the carcinogenic potential of that variant, we could follow the clinical outcomes of women harboring each variant to see if we can correlate the presence of a particular variant or group of variants with the progression of CIN I to CIN III and cervical cancer. A large collaborative project would be necessary.

References

- ¹ Payne, J.F. 1891. Clinical notes on the contagiousness of common warts. *Brit. J. Dermatol.* 3:185-188.
- ² Zur Hausen, H. 2009. Papillomaviruses in the causation of human cancers-a brief historical account. *Virology.* 384:260-265.
- ³ Zur Hausen, H. 1977. Human papilloma viruses and their possible role in squamous cell carcinomas. *Curr. Top. Microbiol. Immunol.* 78:1-30.
- ⁴ Oriel, J.D., and J.D. Almeida. 1970. Demonstration of virus particles in human genital warts. *Brit. J. Vener. Dis.* 46:37-42.
- ⁵ Della Torre, G., Pilotti, S., de Palo, G., and F. Rilke. 1978. Viral particles in cervical condylomatous lesions. *Tumori.* 64:549-553.
- ⁶ Boyle, W.F., Riggs, J.L., Oshiro, L.S., and E.H., Lennette. 1973. Electron microscopic identification of papova virus in laryngeal papilloma. *Laryngoscope* 83:1102-1108.
- ⁷ Dun, A.E., and M.M. Ogilvie, 1968. Intranuclear virus particles in human genital wart tissue: observations on the ultrastructure of the epidermal layer. *J. Ultrastruct. Res.* 22:282-95.
- ⁸ Meisels, A., Fortin, R. and M. Roy. 1977. Condylomatous lesions of the cervix. II. Cytologic, colposcopic and histopathologic study. *Acta. Cytol.* 21:379-390.
- ⁹ Syrjanen, K.J. 1979. Histological and cytological evidence of a condylomatous lesion in association with an invasive carcinoma of uterine cervix. *Arch Geschwulstforsch.* 49:436-443.
- ¹⁰ Levy, J.A., Fraenkel-Conrat, H., and R. A. Owens. 1994. Viruses with small DNA genomes: Papovaviridae. In *Virology*, 3rd ed. Prentice Hall, Englewood Cliffs, New Jersey.
- ¹¹ Narisawa-Saito, M., and T. Kiyono. 2007. Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: Roles of E6 and E7 proteins. *Cancer Sci.* 98:1505-1511.
- ¹² Evander, M., Frazer, I. H., Payne, E., Qi, Y. M., Hengst, K., and N.A.

- McMillan. 1997. Identification of the alpha 6 integrin as a candidate receptor for papillomaviruses. *J. Virol.* 71:2449-2456.
- 13 Chiang, C.M., Ustav, M., Stenlund, A., Ho, T.F., Broker, T.R., and L.T. Chow. 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomavirus origins. *Proc. Natl. Acad. Sci. USA* 89:5799-5803.
- 14 Doorbar, J., Ely, S., Sterling, J., McLean, C., and L. Crawford, L. 1991. Specific interaction between HPV-16 E1-E4 and cytokeratins results and collapse of the epithelial cell intermediate filament network. *Nature* 352: 824-827.
- 15 DiMario, D. and D. Mattoon. 2001. Mechanisms of cell transformation by papillomavirus E5 proteins. *Oncogene* 20:7866-7873.
- 16 Dell, G., and K. Gaston. 2001. Contributions in the domain of cancer research: Review human papillomaviruses and their role in cervical cancer. *Cell. Mol. Life Sci.* 58:1923-1942.
- 17 Zur Hausen, H. 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology.* 184:9-13.
- 18 Howley, P.M. 1996. Papillomavirinae: the viruses and their replication. In *Virology*, B.N. Fields, D.M. Knipe and P.M. Howley, eds., Lippincott-Raven Publishers.
- 19 Cooper, K., Herrington, C.S., Graham, A.K., Evans, M.F., and J.O. McGee. 1991. In situ human papillomavirus genotyping of cervical intraepithelial neoplasia in South African and British patients: evidence for putative HPV integration in vivo. *J. Clin. Pathol.* 44:400-405.
- 20 Angeletti, P.C., Fernandes, F.J., and P.F. Lambert. 2002. Stable replication of papillomavirus genomes in *Saccharomyces cerevisiae*. *J. Virol.* 76:3350-3358.
- 21 Kim, K., and P.F. Lambert. 2002. E1 protein of bovine papillomavirus 1 is not required for the maintenance of viral plasmid DNA replication. *Virol.* 293:10-14.
- 22 Koutsky, L. 1997. Epidemiology of genital human papillomavirus infection. *Am. J. of Med.* 102:3-8.

- 23 McMurray, H.R., Nguyen, D., Westbrook, T.F., and D. J. McAnce. 2000. Biology of human papillomaviruses. *Int. J. Exp. Pathol.* 82:15-33.
- 24 Walboomer, J. M., Jacobs, M. V., Mano, M.M., Bosch, F. X., Kummer, J.A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J., and N. Munoz. 1999. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol*, 189:12-19.
- 25 Bosch, F.X., Manos, M.M., Munoz, N., Sherman, M., Jansen, A.M., Peto, J., Schiffman, M.H., Moreno, V., Kurman, R., and K.V. Shah. 1995. Prevalence of human papillomavirus is a cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) Study Group. *J. Natl. Cancer Inst.* 87:796-802.
- 26 Brentjens, M.H., Yeung-Yue, K.A., Lee, P.C., and S.K. Tyring. 2002. Human papillomavirus: a review. *Dermatol. Clin.* 20:315-331.
- 27 Clifford, G. M. Smith, J. S., Plummer, M., Munoz, N., and S. Franceschi. 2003. Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *Br. J. Cancer* 88:63-73.
- 28 Castle, P.E., Schiffman, M., Wheeler, C.M., and D. Solomon. 2009. Evidence for frequent regression of cervical intraepithelial neoplasia grade 2. *Obstet. Gynecol.* 113:18-25.
- 29 Lawson, H. W., Henson, R., Bobo, J.K, and M.K. Kaeser. 2000. Implementing recommendations for the early detection of breast and cervical cancer among low-income women. *MMWR Recomm. Rep.* 49:37-55.
- 30 Franco, E. L., Duarte-Franco, E., and A. Ferency. 2001. Cervical cancer: an epidemiology, prevention and the role of human papillomavirus infection. *CMAJ* 167:1017-1025.
- 31 Department of Health and Human Services, Division of STD Prevention. 1999. Prevention of genital HPV infection and sequelae: Report of an external consultant's meeting.
- 32 Alexander, K.A., and W.C. Phelps. 2000. Recent advances in diagnosis and therapy of human papillomaviruses. *Exp. Opin. Invest. Drugs* 9:1753-1765.
- 33 Cooper K. and J.O. McGee. 1997. Human papillomavirus, integration and

cervical carcinogenesis: a clinicopathological perspective. *Mol. Pathol.* 50:1-3.

- 34 Schwartz, E., Freese, U. K., Gissmann, L., Mayer, W., Rggenbuck, B., Streamlau, A., and H. zur Hausen. 1985. Structure and transcription of human papilloma virus sequence in cervical carcinoma cells. *Nature* 314:111-114.
- 35 Baker, C. C., Phelps, W.C., Lindgren, V., Braun, M. J., Gonda, M. A., and P.M. Howley. 1987. Structural and transcriptional analysis of human papilloma virus type 16 sequences in cervical carcinoma cell lines *J. Virol.* 61:962-971.
- 36 Hawley-Nelson, P., Vousden, N.L., Hubbert, N. L., Lowy, D.R., and J.T. Schiller. 1989. HPV 16 E6 and E7 proteins cooperate to immortalized human foreskin keratinocytes. *EMBO J.* 8:3905-3910.
- 37 Munger, K., Phelps, W. C., V., Howley P. M., and R. Schlegel. 1989. The E6 and E7 genes of human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.* 63:4417-4421.
- 38 Munger, K., Basile, J.R., Duensing, S., Eichten, A., Gonzalez, S.L., Grace, M., and V.L. Zacny. 2001. Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene* 20:7888-7898.
- 39 Boyer, S. N., Wazer, D. E., and V. Band. 1996. E7 protein of human papillomavirus 16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res.* 56:4620-4624.
- 40 Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and P.M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 63:1129-1136.
- 41 McIntyre, M. C., Ruesch, M. N., and L.A. Laimins. 1996. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. *Virology*, 215:73-82.
- 42 Tommasino, M., Adamczewski, J.P., Carlotti, F., Barth, C.F., Manetti, R., Contorni, M., Cavalieri, F., Hunt, T., and L. Crawford. 1993. HPV 16 E7 protein associates with the protein kinase p33CDK2 and cyclin A. *Oncogene* 8:195-202.

- 43 Jian, Y., Schmidt-Grimminger, D. C., Chien, W. M., Wu, X., Broker, T.R., and L.T. Chow. 1998. Post-translational induction of p21cip1 protein by human papillomavirus E7 inhibits unscheduled DNA synthesis reactivated in differentiating keratinocytes. *Oncogene* 17:2027-2038.
- 44 Jones, D.L., Alani, R.M., and K. Munger. 1997. Human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21cip1-mediated inhibition of cdk2. *Genes Dev.* 11:2101-2111.
- 45 Pim, D., Massimi, P., Dilworth, S. M., and L. Banks. 2005. Activation of the protein kinase B pathway by the HPV 16E7 oncoprotein occurs through a mechanism involving interaction with PP2A. *Oncogene* 24:7830-7838.
- 46 Brazil, D. P., and B.A. Hemmings. 2001. Ten years of protein kinase B signaling: a hard Akt to follow. *Trends Biochem. Sci.* 26:657-664.
- 47 Hubbert, N.L., Sedman, S.A., and J.T. Schiller. 1992. Human papillomavirus type 16 E6 increases the degradation rate of p53 in human keratinocytes. *J. Virol.* 66:6237-6241.
- 48 Huibregtse, J.M., Scheffner, M., and P. Howley. 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Mol. Cell Biol.*, 13:775-784.
- 49 Giarre, M., Calderia, S., Malanchi, I., Ciccolini, F., Leao, M.J. and M. Tommasino. 2001. Induction of pRB degradation by the human papillomavirus type 16 E7 protein is essential to efficiently overcome p16INK4a imposed cell cycle arrest. *J. Virol.* 75:4705-4712.
- 50 Gonzalez S. L., Stremlau, M., He X., Basile, J.R., and K. Munger. 2001. Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for function and activation and is separable from proteasomal degradation of E7. *J. Virol* 75:7583-7591.
- 51 Scheffner, M., Huibregtse, J.M., Vierstra, R.D., and P.M. Howley. 1993. The HPV 16E6 and E6AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 75:495-505.

- 52 Garnett, T.O., Filippova, M., and P.J. Duerksen-Hughes. 2006. Accelerated degradation of FADD and procaspase 8 in cells expressing human papillomavirus 16 E6impairs TRAIL-mediated apoptosis. *Cell Death Differ.* 13:1915-1926.
- 53 Filippova, M., Song, H., Connolly, J.L., Dermody, T.S., and P.J. Duerksen-Hughes. 2004. The human papillomavirus 16 E6 proteins binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J. Biol. Chem.* 277:21730-21739.
- 54 Filippova, M., Parkhurst, L. and P.J. Duerksen-Hughes. 2004. The human papillomavirus 16E6 proteins binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J. Biol. Chem.* 279:25729-25744.
- 55 Patel, D., Huang, S., Baglia, L., and D. McCance. 1999. The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *EMBRO J.* 18:5061-5072.
- 56 Nees, M., Geoghegan, J.M., Hyman, T., Frank, S., Miller, and C.D. Woodworth. 2001. Papillomavirus type 16 oncogenes down regulate expression of interferon-responsive gene and up regulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. *J. Virol.* 75:4283-4296.
- 57 Doyle, D. A., Li, a, Lewis, J., Kim, E., Sheng, M., and R. McKinnon.1996. Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. *Cell* 85:1067-1076.
- 58 Karin, M. Cao, Y., Florian, R., Greten, F.R., and Z.W. Li. 2002. NF-κB in cancer: from innocent bystander to major culprit. *Nature Reviews Cancer* 2:301-310.
- 59 Sovak, M., Bellas, D. Kim, G., Zanieski, A., Rogers, A, Traish, A., and G.E. Sonenshein. 1997. Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. *J. Clin. Invest.* 100:2952-2960.
- 60 Bargou, R., Emmerich, F., Krappmann, D., Bommert, K., Mapara, M., Arnold, W., Royer, H., Grinstein, E., Greier, A., Scheidereit, C., and B. Dorken. 1997. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J. Clin. Invest.* 100:2961-2969.

- 61 Loercher, A., Lee, T.L., Ricker, J.L., Howard, A., Geoghegan, J., Chen, Z., Sunwoo, J.B., Sitcheran, R., Chuang, E.Y., Mitchell, J.B., Baldwin, A.S., and C. Van Waes. 2004. Nuclear factor-kappaB is an important modulator of the altered gene expression profile and malignant phenotype in squamous cell carcinoma. *Cancer Res.* 64:6511-6523.
- 62 Duffey, D., Chen, Z., Dong, G., Ondrey, F., Wolf, J., Brown, K., Siebenlist, U., and Van C. Waes. 1999. Expression of a dominant-negative mutant inhibitor-kappaB alpha of nuclear factor-kappaB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth in vivo. *Cancer Res.* 59:3468-3474.
- 63 Li, Q., and I.M. Verma. 2002. NF- κ B regulation in the immune system. *Nature Rev. Immunol.* 2:725-733.
- 64 Baldwin, A.S. 1996. The NF-kappaB and IkappaB proteins: new discoveries and insights. *Annu. Rev. Immunol.* 14:649-681.
- 65 Jin, D., Giordano, V., Kibler, V., Nakano, H., and K. Jeang. 1999. Role of adaptive function in oncoprotein-mediated activation of NF-kappaB. *J. Biol. Chem.* 18:17402-17405.
- 66 Patel, A., Hanson, J., McLean, T. I., Olgiate, J., Hilton, M., Miller, W. E., and S.L. Bachenheimer. 1998. Herpes simplex type 1 induction of persistent NF-kappaB nuclear translocation increases the efficiency of virus replication. *Virology* 247:212-222.
- 67 Amici, C., Belardo, G. Rossi, A., and M.G. Santoro. 2001. Activation of I kappaB kinase by herpes simplex virus type 1. A novel target for anti-herpetic therapy. *J. Biol. Chem.* 276:28759-28766.
- 68 Gregory, D., Haggatt, D. Holmes, D., Money, E. and S.L. Bacheneimer. 2004. Efficient replication by herpes simplex virus type 1 involves activation of the IkappaB kinase-IkappaB-p65 pathway. *J. Virol.* 78:13582-13590.
- 69 Siebenlist, U., Franzoso, G., and K. Brown. 1994. Structure, regulation and function of NF-kappaB. *Annu. Rev. Cell Biol.* 10:405-455.
- 70 Silverman, N. and T. Maniatis. 2001. NF-kappaB signaling pathways in mammalian and insect innate immunity. *Genes Dev.* 15:2321-2342.
- 71 Grimm, S., and P.A. Baeuerle. 1993. The inducible transcription factor NF-

- kappaB: structure-function relationship of its protein subunits. *Biochem. J.* 290:297-308.
- 72 Miyamoto, S., and I.M. Verma. 1995. Rel/NF-kappa B/I kappa B story. *Adv. Cancer Res.* 65:255-292.
- 73 Deng, L., Wang, C., Spencer, E., Yang, L. Braun, A., You, J., Slaughter, C., Pickart, C., and Z.J. Chen. 2000. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 103:351-361.
- 74 Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Thomas, D., Hay, R.T., Virelizier, J.L., and C. Dargemont. 1997. Nuclear localization of IkappaB alpha promotes active transport of NF-kappaB from the nucleus to the cytoplasm. *J. Cell Sci.* 110:369-378.
- 75 Yamamoto, Y., and R.B. Gaynor. 2004. IkappaB kinases: key regulators of the NF-kappaB pathway. *Trends in Biochemical Sciences*, 29:72-79.
- 76 Karin, M., Yamamoto, Y., and Q.M. Wang. 2004. The IKK NF-kappaB system: a treasure trove for drug development. *Nat. Rev. Drug Discov.* 3:17-26.
- 77 Hacker, H., and M. Karin. 2006. Regulation and function of IKK and IKK-related kinases. 2006 *Sci. STKE.* 357:re13.
- 78 Devin, A., Lin, Y., Yamaoka, S., Li, Z., Karin, M., and Z. Liu. 2001. The alpha and beta subunits of IkappaB kinase (IKK) mediate TRAF2-dependent IKK recruitment to tumor necrosis factor (TNF) receptor 1 in response to TNF. *Mol. Cell. Biol.* 21:3986-3994.
- 79 Agou, Ye, Goffinant, Courtosis. 2000. NEMO trimerizes through its coiled-coil C-terminal domain. *J Biol. Chem.* 277:17464-17475.
- 80 Poyet, J.L., Srinivasula, S.M., Lin, J.H., Fernandes-Alnemri, T., Yamaoka, S., Tichhli, P.N., and E.S. Alnemri. 2000. Activation of the IkappaB kinases by RIP via IKKgamma/NEMO-mediated oligomerization. *J. Biol. Chem...* 275:37966-37977.
- 81 Hu, Y., Baud, V., Oga, T., Kim, K.I. Yoshida, K., and M. Karin. 2001. IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature* 410:710-714.

- 82 Anest, V., Hanson, J.L., Cogswell, P.C., Steinbrecher, K.A., Strahl, B.D., and A.S. Baldwin. 2003. A nucleosomal function for IkappaB kinase-alpha in NF-kappaB-dependent gene expression. *Nature* 423:659-663.
- 83 Bignell, G.R., Warren, W., Seal, S., Takahashi, M., Rapley, E., Barfoot, R., Green, H., Brown, C., Biggs, P.J., Lakhani, S.R., Jones, C., Hansen, J., Blair, E., Hofmann, B., Siebert, R., Turner, G., Evans, D.G., Schrandt-Stumpel, C., Beemer, F.A., van Den Ouweland, A., Halley, D., Delpech, B., Cleveland, M.G., Leigh, I., Leisti, J., and S. Rasmussen, S. 2000. Identification of the familial cylindromatosis tumour-suppressor gene. *Nat. Genet.* 25:160-165.
- 84 D-Andrea, A., and D Pellman. 1998. Deubiquitinating enzymes: a new class of biological regulators. *Crit. Rev. Biochem. Mol. Biol.* 33:337-352.
- 85 Saito, K., Kigawa, T., Koshihara, S., Sato, K., Matsuo, Y., Sakamoto, A., Takagi, T., Shirouzu, M., Yabuki, T., Nunokawa, E., et al. 2004. The CAP-Gly domain of CYLD associates with the proline-rich sequence of NEMO/IKK-gamma. *Structure.* 12:1719-1728.
- 86 Massoumi, R., Chmielarska, K., Hennecke, K., Pfeifer, A., and R. Fassler. 2006. CYLD inhibits tumor cell proliferation by blocking Bcl-3 dependent NF-kappaB signaling. *Cell* 125:665-677.
- 87 Brummelkamp, T.R., Nijman, S.M., Dirac, A.M., and R. Bernards. 2003. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB *Nature* 424, 797-801.
- 88 Trompouki, E., Hatzivassiliou, E., Tschirritzis, T., Farmer, H., Ashworth, A., and G. Mosialos. 2003. CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature* 424:793-796.
- 89 Havard, L., Delvenne, P., Frare, P., Boniver, J., and S.L. Giannini. 2002. Differential production of cytokines and activation of NF-kappaB in HPV-transformed keratinocytes. *Virology.* 298: 271-285.
- 90 Vancurova, I., Wu, R., Miskolci, V., and S. Sun. 2002. Increased p50/p50 NF-kappaB activation in human papillomavirus type 6- or type 11-induced laryngeal papilloma tissue. *J. Virol.* 76:1533-1536.
- 91 Zur Hausen, H. 2000. Papillomaviruses causing cancer: evasion from host-

- cell control in early events in carcinogenesis. *J. Natl. Cancer Inst.* 92:690-699.
- 92 James, M.A., Lee, J.H and A.J. Klingelutz. 2006. Human papillomavirus type16 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner. *J. Virol.* 80:5301-5307.
- 93 Biggs, P.J., Wooster, R., Ford, D., Chapman, P., Mangion, J., Quirk, Y., Easton, D.F., Burn, J., and M.R. Stratton. 1995. Familial cylindromatosis (turban tumour syndrome) gene localised to chromosome 16q12-q13: evidence for its role as a tumour suppressor gene. *Nat. Genet.* 11:441-443.
- 94 Leonard, N., Chaggar, R., Jones, C., Takahashi, M., Nikitopoulou, A., and S.R. Lakhani. 2001. Loss of heterozygosity at cylindromatosis gene locus, CYLD, in sporadic skin adnexal tumours. *J. Clin. Pathol.* 54:689-692.
- 95 Kovalenko, A., Chable-Bessia, C., Cantarella, G., Israel, A., Wallach, D., and G. Courtois. 2003. The tumour suppressor CYLD negatively regulates NF-kappaB signaling by deubiquitination. *Nature* 424:801-805.
- 96 Regamey, A., Hohl, D., Liu, J.W., Roger, T., Kogerman, P., Toftgard, R., and M. Huber. 2003. The tumor suppressor CYLD interacts with TRIP and regulates negatively nuclear factor kappaB activation by tumor necrosis factor. *J. Exp. Med.* 198:1959-1964.
- 97 Baeuerle, P.A., and T. Henkel. 1994. Function and activation of NF-kappaB in the immune system. *Annu. Rev. Immunol.* 12:141-179.
- 98 Karin, M., Cao, Y., Greten, F.R., and Z.W. Li. 2002. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat. Rev. Cancer* 2:301-310.
- 99 Balkwill, F., and L.M. Coussens. 2004. Cancer: an inflammatory link. *Nature* 431:405-406.
- 100 Coussens, L.M., and Z. Werb. 2002. Inflammation and cancer. *Nature* 420: 860-867.
- 101 Schottenfeld, D., and J. Beebe-Dimmer. 2006. Alleviating the burden of cancer: a perspective on advances, challenges, and future directions. *Cancer Epidemiol. Biomarkers Prev.* 15:2049-2055.
- 102 Mueller, M.M. 2006. Inflammation in epithelial skin tumours: old stories and

new ideas. *Eur. J. Cancer* 42:735-744.

- 103 Jeon, S., Allen, H. B., and P. F. Lambert. 1995. Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *J. Virol.* 69:2989-2997.
- 104 Flores, E.R., and P.F. Lambert. 1997. Evidence for switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. *J. Virol.* 71:7167-7179.
- 105 Woodworth, C.D. 2002. HPV innate immunity. *Front. Biosci.* 7:d2058 d2071.
- 106 Hochstrasser, M. 1996. Protein degradation or regulation: Ub the judge. *Cell.* 84:813-815.
- 107 Yang, Y., Kitagaki, J., Wang, H., Hou, D., and A.O. Pertantoni. 2009. Targeting the ubiquitin-proteasome system for cancer therapy. *Cancer Sci.* 100:24-28.
- 108 Coscoy, L., Sanchez, D.J., and D. Ganem. 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J. Cell Biol.* 155:1265-1273.
- 109 Harada, J.N., Shevchenko, A., Shevchenko, A., Pallas, D.C., and A.J. Berk. 2002. Analysis of the adenovirus E1B-55k-anchored proteome reveals its link to ubiquitination machinery. *J. Virol.* 76:9194-9206.
- 110 Querido, E., Blanchette, P., Tan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W.G., Conaway, R.C., Conaway, J.W., and P.E. Branton. 2001. Degradation of p53 by adenovirus E4orf6 and E1B55k proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev.* 15:3104-3117.
- 111 Baker, A., Rohleder, K.J., Hanakahi, L.A., and G. Ketner. 2007. Adenovirus E4 34K and E1b55k oncoproteins target hot DNA ligase IV for proteasomal degradation. *J. Virol.* 81:7034-7040.
- 112 Chaturvedi, V., Qin, J.Z., Denning, M.F., Choubey, D., Diaz M.O., and B.J. Nickoloff. 1999. Apoptosis in proliferating, senescent, and immortalized keratinocytes. *J. Biol. Chem.* 274:23358-23367.

- 113 Berberich, I., Shu, G.L., and E.A. Clark. 1994. Cross-linking CD40 on B cells rapidly activates nuclear factor-kappaB. *J. Immunol.* 153:4357-4366.
- 114 Coope, H.J., Atkinson, P.G.P., Huhse, B., Belich, M., Janzen, J., Holman, M.J., Klaus, G.G.B., Johnston, L.H., and S.C. Ley. 2002. CD40 regulates the processing of NF-kappaB2 p100 to p52. *EMBO J.* 21:5375-5385.
- 115 Kosaka, Y., Calderhead, D.M., Manning, E.M., Hambor, J.E., Black, A., Geleziunas, R., Marcu, K.B., and R.J Noelle. 1999. Activation and regulation of the IkappaB kinase in human B cells by CD40 signaling. *J. Immunol.* 29:1353-1362.
- 116 Munger, K., and P.M. Howley. 2002. Human papillomavirus immortalization and transformation functions. *Virus Res.* 89:213-228.
- 117 Zur Hausen, H. 2002. Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer* 2:342-350.
- 118 Arch, R.H., Gedrich, R.W., and C.B. Thompson. 1998. Tumor necrosis factor receptor-associated factors (TRAFs)-a family of adaptor proteins that regulate life and death. *Genes Devel.* 12:2821-2830.
- 119 Bradley, J.R., and S.P. Jordan. 2001. Tumor necrosis factor receptor-associated factors (TRAFs). *Oncogene* 20:6482-6491.
- 120 Reiley, W., Zhang, M., Wu, X., Granger, E., and S. Sun. 2005. Regulation of the deubiquitinating enzyme CYLD by IkappaB kinase gamma-dependent phosphorylation. *Mol. Cell. Bio.* 25:3886-3895.
- 121 Hall, A.H.S., and K.A. Alexander. 2003. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. *J Virol.* 77:6066-6069.
- 122 Hellerbrand, C., Bumes, E., Bataille, F., Dietmaier, W., Massoumi, R., and A.K. Bosserhoff. 2007. Reduced expression of CYLD in human colon and hepatocellular carcinomas. *Carcinogenesis.* 28:21-27.
- 123 Lee, S.Y., Lee, S.Y, and Y. Choi. 1997. TRAF-interacting protein (TRIP): A novel component of tumor necrosis factor receptor (TNFR)- and CD30-TRAF signaling complexes that inhibits TRAF2-mediated NF-kappaB activation. *J. Exp. Med.* 185:1275-1285.

- 124 Hellerbrand, C., Bumes, E., Bataille, F., Dietmaier, W., Massoumi, R., and A.K. Bosserhoff. 2007. Reduced expression of CYLD in human colon and hepatocellular carcinomas. *Carcinogenesis*. 28:21-27.
- 125 Bosch, F.X., Manos, M.M., Munoz, N., Sherman, M., Jansen, A.M., Peto, J, et al. 1995. Prevalence of human papillomavirus DNA in cervical cancer: a worldwide perspective. International Biological study on cervical cancer study group. *J. Natl. Cancer Inst.* 11:796-802.
- 126 Laimins, LA. 1993. The biology of human papillomaviruses: from warts to cancer. *Infect. Agents Dis.* 2:74-86.
- 127 Dalal, S., Gai, Q., Androphy, E.J., and V. Band. 1996. Mutational analysis of human papillomavirus type 16 E6 demonstrates that p53 degradation is necessary for immortalization of mammary epithelial cells. *J. Virol.* 70:683-688.
- 128 Barbosa, M.S., Lowy, D.R., and J.T. Schiller. 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *J. Virol.* 63:1404-1407.
- 129 Lipari, F., McGibbon, G.A., Wardrop, E., and M.G. Cordingley. 2001. Purification and biophysical characterization of a minimal functional domain and of an n-terminal Zn²⁺-binding fragment from the human papillomavirus type 16 E6 protein. *Biochemistry* 40:1196-1204.
- 130 Yamada, T., Wheeler, C.M., Halpern, A.L., Stewart, A.C., Hildesheim, A., and S.A. Jenison. 1995. Human papillomavirus type 16 variant lineages in United States populations characterized by nucleotide sequence analysis of the E6, L2, and L1 coding segments. *J. Virol.* 69:7743-7753.
- 131 Zehbe, I., Wilander, E., Delius, H., and M. Tommasino. 1998. Human papillomavirus 16E6 variants are more prevalent in invasive cervical carcinoma than the prototype. *Cancer Research* 58:829-833.
- 132 Bulkman, N.W., Berkhof, J., Bulk, S., Bleeker, M.C., van Kemenade, F.J., Rozendaal, L., Snijders, P.J., Meijer, C.J., POBASCAM Study Group. 2007. High-risk HPV type-specific rates in cervical screening. *Br. C. Cancer.* 96:1419-1424.
- 133 Rosa, M.I., Fachel, J.M., Rosa, D.D., Medeiros, L.R., Igansi, C.N., and M.C.

- Bozzetti. 2008. Persistence and clearance of human papillomavirus infection: a prospective cohort study. *Am. J. Obstet. Gynecol.* 199: 617.e1-617.e7.
- 134 Palefsky, J. 2006. Biology of HPV in HIV infection. *Adv. Dent. Res.* 19:99-105.
- 135 Sun, X.W., Ellerbrock, T.V., Lung, U., Chiasson, M.A., Bush, T.J and T.C Wright. 1995. Human papillomavirus infection in human immunodeficiency virus-seropositive women. *Obstet. Gynecol.* 85:680-686.
- 136 Duerr, A., Kieke, B., Warren, D., Shah, K., Burk, R., Piepert, J.F., Schuman, P., Klein, R.S., and HER Study group. 2001. Human papillomavirus-associated cervical cytologic abnormalities among women with or at risk of infection with human immunodeficiency virus. *Am. J. Obstet. Gynecol.* 184:584-590.
- 137 Durante, A.J., Williams, A.B., Da Costa, M., Darragh, T.M., Khoshnood, K., and J.M. Palefsky. 2003. Incidence of anal cytological abnormalities in a cohort of human immunodeficiency virus-infected women. *Cancer Epidemiol. Biomarkers Prev.* 12:638-642.
- 138 Melbye, M., Smith, E., Wohlfahrt, J., Osterlind, A., Orholm, M, Bergmann, O.J., Mathiesen, L., Darragh, T.M., and J.M. Palefsky. 1996. Anal and cervical abnormality in women-predictions by human papillomavirus tests. *Int. J. Cancer* 68:559-564.
- 139 Kreimer, A.R., Alberg, A.J., Daniel, R., Gravitt, P.E., Viscidi, R., Garrett, E.S., Shah, K.V., and M.L. Gillison. 2004. Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. *J. Infect. Dis.* 189:686-698.
- 140 Sun, X.W., Kuhn, L., Ellerbrock, T.V., Chiasson, M.A., Bush, T.J., and T.C. Wright, Jr. 1997. Human papillomavirus infection in women infected with the human immunodeficiency virus. *N. Engl. J. Med.* 337:134-139.
- 141 Chaturved, A.K., Brinkman, J.A., Gaffga, A.M., Dumestre, J., Clack, R.A., Braly, P.S., Dunlap, K., Kissinger, P.J., and M.E. Hagensee. 2004. Distribution of human papillomavirus type 16 variants in human immunodeficiency virus type 1-positive and -negative women. *J. Gen. Virol.* 85:1237-1241.
- 142 Perez-Gallego, L., Moreno-Bueno, G., Sarrío, D., Suarez, A., Gamallo, C.,

- and J. Palacios. 2001. Human papillomavirus 16E6 variants in cervical squamous intraepithelial lesions from HIV-negative and HIV-positive women. *Am. J. Clin. Pathol.* 116:143-148.
- 143 Da Costa, M.M., Hogeboom, C.J., Holly, E.A., and J.M. Palefsky 2002. Increased risk of high grade anal neoplasia associated with human papillomavirus type 16 E6 sequence variant. *Journal of Infectious Diseases.* 185:1229-1237.
- 144 Qui, A., Wu, E., Yu, X., Jiang, C., Jin, Y., Wu, Y., Chen, Y., Chen, Y., Shan, Y., Zhang, G., Fan, Y., Zha, X., and W. Kong. 2007. HPV prevalence, E6 sequence variation and physical state of HPV16 isolates from patients with cervical cancer in Sichuan, China. *Gyn. Oncol.* 104:77-85.
- 145 Zehbe, I., Wilander, E., Delius, H., and M. Tommasino. 1998. Human papillomavirus 16E6 variants are more prevalent in invasive cervical carcinoma than the prototype. *Cancer Research* 58:829-833.
- 146 Grodzki, M., Besson, G., Clavel C., Arslan, A., Franceschi, S., Birembaut, P., Tommasino, M., and I. Zehbe. 2006. Increased risk for cervical cancer disease progression of French women infected with the human papillomavirus type 16 E6-350G variant. *Cancer Epidemiol. Biomark. Prev.* 15:820-822.
- 147 Chakrabarti, O., Veeraraghavalu, K., Tergaonkar, V., Lu, Y., Androphy, E.J., Stanley, M.A., and S. Krishna. 2004. Human papillomavirus type E6 amino acid 83 variant enhances E6-mediated MAPK signaling and differentially regulate tumorigenesis by notch signalling and oncogenic Ras. *J. Virol.* 78:5934-5945.
- 148 Lee, K., Magalhaes, I., Clavel, C., Briolat, J., Birembaut, P., Tommasino, M., and I. Zehbe. 2008. Human papillomavirus 16E6, L1, L2, and E2 gene variants in cervical lesion progression. *Virus Research.* 131:106-110.
- 149 Liu, Y., Chen, J.J., Gao, Q., Dalal, S., Hong, Y., Mansur, C.P., Band, V., and Androphy, E.J. 1999. Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. *J. Virol.* 73:7297-7307.
- 150 Thomas, M., and L. Banks. 1999. Human papillomavirus E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *J. General Virol.* 80:1513-1517.

- 151 Thomas, M., and L. Banks. 1998. Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* 17:2943-2954.
- 152 Garnett, T.O., Filippova, M., and P.J. Duerksen-Hughes. 2006. Accelerated degradation of FADD and procaspase 8 in cells expressing human papillomavirus 16, E6 impairs TRAIL-mediated apoptosis. *Cell Death Differ.* 13:1915-1526.
- 153 Kuner, R., Vogt, M., Sultmann, H., Buness, A., Dymalla, S., Bulkeschen, J., Fellmann, M., Butz, K., Poustka, A., and F. Hoppe-Seyler. 2007. Identification of cellular targets for the human papillomavirus E6 and E7 oncogenes by RNA interference and transcriptome analysis. *J. Mol. Med.* 85:1253-1262.
- 154 Lui, X., Dakic, A., Chen, R., Disbrow, G.L., Zhang, Y., Dai, Y., and R. Schlegel. 2008. Cell-restricted immortalization by human papillomavirus correlates with telomerase activation and engagement of the hTERT promoter by Myc. *J. Virol.* 82:11568-11576.
- 155 Watson, R.A., Thomas, M., Banks, L., and S. Roberts. 2003. Activity of the human papillomavirus E6 PDZ-binding motif correlates with an enhanced morphological transformation of immortalized human keratinocytes. *J. Cell Sci.* 116:4925-4934.
- 156 James, M.A., Lee, J.H., and A.J. Klingelutz. 2006. Human papillomavirus type 16 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner. *J. Virol.* 80:5301-5307.
- 157 Chen, J.J., Hong, Y., Rustamzadeh, E., Baleja, J.D., and E.J. Androphy. 1998. Identification of an alpha helical motif sufficient for association with papillomavirus E6. *J. Biol. Chem.* 273:13537-13544.
- 158 Ikeda, F., and I. Dikic. 2006. CYLD in ubiquitin signaling and tumor pathogenesis. *Cell* 125:643-645.
- 159 Lakhani, S.R. 2004. Putting the brakes on cylindromatosis. *New Engl. J. Med.* 350:187-188.
- 160 Zanier, K., Charbonnier, S., Baltzinger, M., Nomine, Y., Altschuh, D., and G.

- Trave. 2005. Kinetic analysis of the interactions of human papillomavirus E6 oncoproteins with the ubiquitin ligase E6AP using surface plasmon resonance. *J. Mol. Biol.* 349:401-412.
- ¹⁶¹ An, J., Mo, D., Liu, H., Veena, M.S., Srivatsan, E.S., Massoumi, R., and M.B. Rettig. Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-kappaB activation. *Cancer Cell* 14:394-407.
- ¹⁶² Balkwill, F., and L.M. Coussens. 2004. Cancer: an inflammatory link. *Nature* 431:405-406.
- ¹⁶³ Mueller, M. 2006. Inflammation in epithelial skin tumors: old stories and new ideas. *Europ. J. Cancer* 42:735-744.
- ¹⁶⁴ Castle P.E Hiller, S.L., Rabe, L.K., Hildesheim, A., Herrero, R., Bratti, M.C., Sherman, M.E., Burk, R.D., Rodriguez, A.C, Alfaro, M., Hutchinson, M.L., Morlaes, J., and M. Schiffman. 2001. An association of cervical inflammation with high-grade cervical neoplasia in women infected with oncogenic human papillomavirus. *Cancer Epide. Biomark. Prev.* 10:1021-1027.
- ¹⁶⁵ Lehtinen, M., Luukkaala, T., Wallin, K.L., Paavonen, J., Thoresen, S., Dillner, J., and M. Hakama. 2001. Human papillomavirus infection, risk for subsequent development of cervical neoplasia and associated population attributable fraction. *J. Clin. Virol.* 22:117-124.
- ¹⁶⁶ Sun, X.W., Kuhn, L., Ellerbrock, T.V., Chiasson, M.A., Bush, T.J., and T.C Wright Jr. 1997. Human papillomavirus infection in women infected with the human immunodeficiency virus. *N. Engl. J. Med.* 337:134-139.
- ¹⁶⁷ Maiman, M., Fruchter, R.G., Sedlis, A., Feldman, J., Chen, P., Burk, R.D., and H. Minkoff. 1998. Prevalence, risk factors, and accuracy of cytologic screening for cervical intraepithelial neoplasia in women with the human immunodeficiency virus. *Gynecol. Oncol.* 68:233-239.
- ¹⁶⁸ Bernard, H.U., Calleja-Macias, I.E., and S.T. Dunn. 2006. Genome variation of human papillomavirus types: phylogenetic and medical implications. *Int. J. Cancer* 118:1071-1076.
- ¹⁶⁹ Stoppler, M.C., Ching, K., Stoppler, H., Clancy, K., Schlegel, R., and J.

Icenogle. 1996. Natural variants of the human papillomavirus type 16 E6 protein differ in their abilities to alter keratinocyte differentiation and to induce p53 degradation. *J. Virol.* 70:6987-6993.

Biography

Charlie Vincent Shaw Junior was born in Fayetteville, North Carolina on December 22, 1978. Charlie Shaw attended Davidson College from 1997 to 2001 and earned a Bachelors of Science in Biology. He was the recipient of a Virology Training Fellowship (2003-2004), Duke Society Fellowship (2001-2005), and the Ruth L. Kirschstein National Research Service Award (2004-2005). He was selected as a National Institutes of Health, NIAID Division, Research Opportunities Program Awardee in 2007.